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TENNESSEE VALLEY AUTHORITY
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EXECUTIVE SUMMARY

The coal ash spill on December 22, 2008 at the TVA Kingston Fossil Plant posed health risks to a wide variety of fish and wildlife. The event discharged approximately 5.4 million cubic yards of coal ash into the nearby Emory River, resulting in an unprecedented remediation effort by TVA. Because coal ash can contain elevated concentrations of a wide variety of potentially toxic trace elements, including arsenic, cadmium, selenium, strontium, and vanadium, TVA initiated monitoring efforts of tissue concentrations in invertebrates, fish, and wildlife that could be affected by the spill. However, more detailed effects-based studies were also necessary to determine whether TVA's remediation efforts were effective. It is possible that the health of fish and wildlife is compromised from their exposure to fly ash following the spill, the physical disturbance of the spill, and/or the continued exposure to residual fly ash and associated trace elements that remain in the river system. Thus, our efforts are focused on providing a comprehensive, post-remediation assessment of aquatic and terrestrial wildlife health in the area. We specifically concentrate on quantifying physiological, behavioral, and reproductive effects on turtles and insectivorous birds because both are known to be useful for large scale ecological assessments of environmental damage and have been used in the NRDA process. This 2012-2013 Annual Report represents the second in a series of three annual reports that will provide TVA with a detailed analysis of wildlife health in the Kingston area. Only 2011 trace element data were available to us prior to report submission, so all comparisons described below are restricted to site-specific comparisons and 2011 trace element data. Final conclusions from the two field seasons (2011 and 2012) will be possible once tissue concentrations of trace elements are available for interpretation.

Turtles are ideal study species for assessing and monitoring the effects of the Kingston, TN coal ash spill because of their natural history characteristics, life history traits, and experimental tractability. Many turtle species are long-lived, feed in the benthos, and have small home ranges, making them excellent model species for long term monitoring and determining spatial distributions of effects of contaminants derived from coal fly ash. Large numbers of turtles and turtle eggs can be easily collected from the field and maintained in captivity, which allows them to be excellent subjects for both field and laboratory research. Our two field seasons (summer 2011 and 2012) were an enormous success. In total, we captured 9,396 turtles representing seven different species from sites on the Clinch, Emory, and Tennessee Rivers. We measured individuals, sampled their tissues for trace element analyses, and gave turtles a unique ID for ongoing mark-recapture efforts. We found no differences in turtle body size, species composition, or relative abundance (based on capture rates) among river locations that were likely attributable to the spill. To assess reproductive health, we collected eggs from gravid females and artificially incubated them in the laboratory to assess maternal transfer of trace elements (e.g., Se) and resultant effects on early development (results to be reported next year). We established that removing one turtle egg from the clutch for trace element analyses was a good indication of concentrations in other eggs within the same clutch. In conjunction with stable isotope analyses to infer relative aspects of feeding ecology, we also demonstrated that trace element accumulation differed among turtle species in this freshwater community. Specifically, we found that different turtle species were feeding across more than one trophic level, and that different turtle species occupied food chains that differed in their relative proportions of C3 and C4 basal producers. Contrary to our predictions, however, there was no relationship between relative trophic position and Se concentrations in turtles. Instead, relative carbon source appeared to be more important than trophic level in determining Se accumulation in turtles.

Although turtles are exposed to trace elements within their aquatic habitats downstream from the ash spill, terrestrial consumers can also be exposed when they drink water or ingest prey that emerge from contaminated areas. Tree swallows (*Tachycineta bicolor*) are one of the primary model species used in North America to address

the movement of contaminants from aquatic to terrestrial ecosystems. They are aerial insectivores that commonly eat emerging aquatic insects, making them susceptible to exposure to residual trace elements that may remain in the Kingston system following remediation. Because tree swallows are secondary cavity nesters and readily use nest boxes, we were able to strategically establish nine colonies (nearly 500 nest boxes) spread across impacted and reference areas. Over two field seasons (2011 and 2012), we used these colonies to determine if tree swallows breeding around Kingston are being exposed to elevated concentrations of trace elements, if those elements are maternally transferred to offspring during egg production, and/or if adults trophically transfer elements to their offspring during nestling provisioning. We are also examining aspects of reproductive success such as clutch size, hatching success, and fledging success to determine if these were affected by exposure to trace elements and/or the physical disturbances associated with the spill and remediation efforts. Finally, we also quantify more subtle effects that trace element exposure could have on nestling quality by examining parameters such as nestling body size, condition, and physiological parameters (e.g., endocrine and immune responses) in impacted and reference colonies.

In our first field season (2011), approximately 77% of our nest boxes were occupied by swallows and we individually banded 1,544 swallows. In 2012, next box occupancy increased to 87% and we banded an additional 2,513 individuals. This remarkable sampling effort was intended to enable us to monitor return rates of individuals, a very sensitive and integrative measure of swallow performance and health that requires robust sample sizes. However, using return rates to estimate bird survival requires at least three years of mark recapture data, and construction of the park on Emory River Peninsula in spring/summer 2013 prevented us from being able to address this important question. However, our robust sample sizes will enable us to draw strong conclusions regarding effects other than survival in this system.

Based on two years of data, clutch size was similar among colonies, averaging ~ 5 eggs/ clutch. Several trace elements were transferred to eggs in significantly higher concentrations at the spill site compared to reference sites including Hg, Se, Sr, and Tl. However, concentrations of embryotoxic elements like Hg and Se were below levels typically associated with reduced hatching success in birds. Consistent with these modest concentrations, hatching success was over 80% at all colonies, and did not differ significantly among colonies. After hatching, adult swallows provisioned their young with a wide array of prey items. Of the 3,675 invertebrates identified in food bolus samples being offered by adults to their young in 2011 (footnote: we collected three times this many boluses in 2012 which are currently being dissected), approximately 67% had an aquatic stage in their life cycle, confirming the important trophic connection between swallows and the nearby aquatic environment. Dipterans were the most commonly encountered prey item, comprising ~78% of the total number of insects collected and present in over 90% of the bolus samples. Concentrations of several trace elements were elevated in insect bolus samples near the spill site including Ba, Cu, Cd, Fe, Mn, Sr, Se, and Zn. However, when only single elements are considered (i.e., potential interactions are not accounted for) these concentrations were generally below the dietary concentrations associated with adverse effects in fish and wildlife. Consistent with these findings, nestlings had higher concentrations of several trace elements in their blood than nestlings from reference colonies. Importantly, we found that nestlings in the spill area were generally in good health (e.g., body size, body condition) and they were just as likely to survive to fledging (day 17) as nestlings from reference colonies.

Taken together, our two field seasons produced an array of valuable insights that will be critical towards our comprehensive analysis of wildlife health in the Kingston area. Although it is premature to draw firm conclusions in the absence of the 2012 trace element data and forthcoming physiological data, our field observations provide a preliminary, positive outlook that suggests many species of wildlife may currently experience limited adverse effects in the Kingston area following TVA's remediation efforts. Turtles and birds appear to produce similar numbers of

offspring, but continuing measures of animal health and performance (e.g., measures of immunocompetence) and the 2012 analytical data will allow us to finalize our conclusions. Taken together, our initial assessment suggests that TVA's rapid and extensive remediation efforts, coupled with the large dilution factor in this lotic system, may have prevented some of the long-term ecological damage that could have been possible following such a large-scale release of ash. Our continuing efforts at the site will provide TVA with a highly integrative, comprehensive, and conclusive ecological assessment, and will assist them in identifying priorities for long-term monitoring at the site.

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INTRODUCTION

Coal burning power plants generate 40% of the electricity produced globally and over half of the electricity produced in the United States each year (IEA, 2012). Global electricity demand is expected to double by 2030 and the use of coal to produce electricity is projected to increase to help meet this demand (IEA, 2012). The United States contains over a quarter of the global coal reserves and over one million tons of coal are mined each year to support domestic and international demands (USEIA, 2012). However, mining, transporting, and burning coal are associated with a variety of environmental and human health risks (Epstein et al., 2011). For instance, coal combustion produces over 130 million tons of waste each year in the United States (ACAA, 2011) and disposing of this quantity of material while mitigating environmental risks poses logistical difficulties (NRC, 2006). Some of this material is recycled or used as fill material in old coal mines but much of it is stored in aquatic impoundments (ACAA, 2011; NRC, 2006; Rowe et al., 2002). Coal-fly ash is one of the primary components of combustion waste and when stored in aquatic impoundments, trace elements and metals may leach into the water and potentially contaminate surface and ground water. Elevated concentrations of trace elements such as arsenic (As), cadmium (Cd), mercury (Hg), and selenium (Se) are often detected and pose health risks to humans and wildlife (NRC, 2006; Rowe et al., 2002). Research is needed to address the environmental impacts of aquatic impoundments and other disposal methods in order to identify the disposal practice that best mitigates these risks.

Several of the elements that leach from coal fly-ash stored in aquatic impoundments are associated with reproductive impairment, reduced survival, and teratogenicity in avian species. Exposure to contaminants such as Cd, Se, and As can reduce egg size, egg shell strength, and induce egg laying gaps within clutches in European songbirds (Eeva and Lehikninen, 1995, 2010; Nyholm and Myhrberg, 1977). Nestlings raised in areas polluted by heavy metals may exhibit poor body condition which reduces their likelihood of surviving after fledging (Janssens et al., 2003). Many of these studies focus primarily on dietary exposure of adults and nestlings but some trace elements can also be maternally transferred to eggs, providing an additional exposure route for developing young (Brasso et al., 2010; Bryan et al., 2003; Harding, 2008; Weech et al., 2012). Maternally transferred trace elements, such as Se and Hg, can reduce hatching success and cause severe nestling malformations (Burger and Gochfield, 1997; Heinz and Hoffman, 2003; Ohlendorf, 2011).

In December 2008, a coal-fly ash impoundment at the Tennessee Valley Authority fossil plant in Kingston, TN ruptured releasing 4.13 million m^3 of coal-fly ash slurry into the Emory River, which then flowed into the Clinch and Tennessee Rivers (TVA, 2009). In the 2.5 years since the spill, most of the coal-fly ash has been removed from the river system but approximately 400,000 m^3 remains in the system (TVA, 2011b). To determine if remediation efforts have reduced risks to wildlife, we examined the effects of residual trace element contamination on reproduction, nestling quality, and recapture rates of a terrestrial consumer, the Tree Swallow (*Tachycineta bicolor*). Tree Swallows are one of the primary model species used to address the movement of contaminants from aquatic to terrestrial ecosystems because they are aerial insectivores and, when breeding in riparian areas, primarily feed on emerging aquatic insects (Beck et al., In review; Custer, 2011; Custer et al., 2010). This makes Tree Swallows susceptible to trace element exposure and because they are secondary cavity nesters (Robertson et al., 2011), nest box colonies can be strategically placed in exposed and reference areas. Both sexes remain close to their nest site throughout the breeding season and typically forage within 300-500 m of their box (Dunn and Hannon, 1992; Quinney and Ankney, 1985) ensuring that they are exposed to local contaminants.

The purpose of this study was to determine if Tree Swallows breeding at the Kingston spill site are exposed to elevated concentrations of trace elements and if those elements are maternally or trophically transferred to offspring

during egg production or nestling provisioning, respectively. We examined adult and nestling recapture rates and the effects of harsh weather and trace element exposure on rates of nest failure. We predicted that birds exposed to elevated trace element concentrations would be less likely to be recaptured and would be more strongly affected by an additional source of environmental stress such as severe weather. We also examined clutch size, hatching, and fledging success to determine if reproductive success was affected by exposure to trace elements. We compared nestling body size and condition among colonies in order to detect more subtle effects of trace elements on nestling quality. We predicted that reproductive success would be lower and nestlings would be smaller and in worse condition at colonies highly impacted by the spill.

METHODS

FIELD METHODS

We studied Tree Swallows in Roane and Loudon Counties, TN, from March-July 2011 and 2012. Our study site encompassed a trace element contamination gradient that ranged from background exposure at reference colonies to potentially high exposure near the site of the ash spill and in areas where ash was not removed during remediation efforts (Figure 1, Table 1). We placed nest boxes in two colonies along the Emory River (Spill Site and Downstream 1), two colonies along the Clinch River (Downstream 2 and Downstream 3), and one colony downstream on the Tennessee River, Downstream 4. We had three reference colonies; two located approximately 30.5 km east of Kingston at Ft. Loudoun Dam (Reference 1) and at Tellico Dam (Reference 2). Reference 3 was located upstream on the Tennessee River from the confluence with the Clinch River, at Long Island. We also placed boxes at Melton Hill Dam on the Clinch River which served a role analogous to a positive control because preliminary data gathered prior to this study indicated that Tree Swallows are exposed to ash-related contaminants such as Se at this colony (ARCADIS, 2011). The source(s) of this contamination is unclear, but could include a former coal ash storage pond associated with the Y-12 Security Complex (Cook et al., 1999), the Bull Run Fossil Plant (Stantec, 2009; TVA, 2011a), and/or other non-point source pollution (USDA, 2009) and warrants further investigation. All of the colonies were established at least one year prior to this study except for Downstream 1 which we established in 2011. We also substantially altered the arrangement of nest boxes at Reference 3 due to box flooding in the previous year.
We placed or maintained nest boxes in each area in late February or early March, when Tree Swallows were arriving and prospecting for nest sites. All of the boxes were located within 70 m of the shore to facilitate foraging on emerging aquatic insects. We spaced nest boxes 15 m apart when in a single row, or 20 m apart with a staggered alignment when there were two or more rows. We began checking nest boxes every four days in late March for signs of nesting activity so that we could accurately record clutch initiation dates. We recorded the final clutch size, number of eggs that hatched, and the number of young that survived to day 17 (adjusted for eggs that were removed for contaminant analysis, see below).

We captured adults using mist nets or trapped them in the nest box while incubating or provisioning young. Adults were sexed by the presence of a brood patch (females) or cloacal protuberance (males) or, if these were absent, by measuring the wing length, with wings shorter than 113 mm being indicative of females and wings longer than 122 mm being indicative of males (Stutchbury and Robertson, 1987). Females were also aged as second year (SY) or after-second year (ASY) based on plumage coloration; SY females were primarily brown and ASY females were iridescent blue. Adults were banded with a combination of one metal band and one colored leg band placed on opposite legs to help us distinguish individuals breeding in neighboring boxes and between males and females from a distance. We obtained small ($\leq$ 120 μl) blood samples from all adults by puncturing the brachial vein after

cleansing the area with 90% ethanol. Sixty microliters of whole blood were frozen for trace element analysis and the remaining blood was stored in lysis buffer. From each adult, we measured bill length, width, and depth, left and right tarsus length (each tarsus was measured twice), wing chord, tail length, and body mass. When nestlings were 13 days old, we obtained morphological measurements (as described for adults), banded them with a metal leg band, and obtained a small blood sample ($\leq$ 120 μl).

Unseasonably cold weather in 2011 allowed us to opportunistically address the potential additive or synergistic effect of extreme weather and trace element exposure on reproductive success in tree swallows. The normal average high temperature in Kingston from May 12th –May 19th is 77-78°F (25-25.5°C). In 2011, from May 12th–May 14th, the average high temperature was 81°F (27.2°C) but on May 15th, a cold front moved through the area and the average high temperature from May 15th-May 18th was only 58.5°F (14.7°C) with an average low temperature of 49.5°F (9.7°C). The high temperature on May 19th returned to 74°F (23.3°C) which marked the end of the four day cold-snap. Additionally, May 15th and May 18th had periods of drizzle but total precipitation on these days was less than 0.13 cm. We categorized nests as affected or unaffected by this weather event. Nests were considered affected if one or more nestling(s) were found dead in the box or if partial brood loss occurred (part of the brood disappeared from the nest box) from May 15th-May 19th. We also considered nests affected by the cold-snap if complete hatching failure occurred in clutches scheduled to hatch May 15th-May 21st. Nests were considered unaffected by the cold-snap if all of the nestlings present in the brood on May 15th survived through to May 19th or if at least one egg hatched as expected.

MATERNAL TRANSFER OF TRACE ELEMENTS

We assessed maternal transfer of trace elements to eggs by collecting a single egg randomly from each clutch within two days of clutch completion. Eggs were placed in plastic bags, labeled with a unique sample id, and protected in plastic containers lined with bubble wrap. Samples were placed in a cooler until transported back to the lab, where eggs were weighed to the nearest 0.001g and the length and width were recorded. Eggs were frozen and stored at -20°C until being prepared for trace element analysis. We also wanted to determine if female blood trace element concentrations were indicative of egg concentrations. To do this, we obtained blood samples from a subset of females (N = 40) within 7 days of clutch initiation to compare to egg concentrations.

SAMPLE PREPARATION AND TRACE ELEMENT ANALYSIS

Egg samples were prepared for trace element analysis at Virginia Tech. Eggs were slightly thawed and a single incision was made in the shell and all shell fragments were removed and returned to the plastic storage bag. The still frozen egg contents (yolk and albumin) were transferred to a 5 ml plastic container and weighed to the nearest 0.0001g. Eggs were placed in the -80°C freezer for 20 min until samples were frozen solid and then dried to asymptotic mass in a freeze drier. After drying, eggs were homogenized by stirring vigorously with a Teflon spatula that was cleaned between samples with metal-free detergent (Citranox®) and Millipore water and dried with a Kim Wipe. We transferred 50-100 mg of homogenized egg to a pre-weighed eppendorf tube and samples were stored in a dessicator until being shipped for trace element analysis.

Samples were shipped overnight on dry ice to the Environmental Analytical Chemistry Lab at Dartmouth University. Concentrations of trace elements present in blood and egg samples were quantified using Inductively Coupled Mass Spectrometry (ICP-MS). In 2011, Concentrations of Al, Sb, As, Ba, Be, B, Cd, Cr, Co, Cu, Fe, Pb, Mn, Hg, Mo, Ni, Se, Sr, Tl, V, and Zn were quantified for each sample. However, Al, Sb, Be, B, Co, Pb, Mo, Ni, and Ag

concentrations were not used it statistical analyses because they were not found in concentrations of toxicological concern, were analytically problematic, or were consistently below the detection limit. However, we present means ± 1 SE in tissues where these elements were detectable in > 50 % of samples. Samples were digested by open vessel digestion with 0.5 ml 9:1 HNO_3:HCl (Optima, Fisher Scientific, St Louis MO) using microwave heating at 105°C for 45 minutes. After cooling, 0.1 ml H_2O_2 was added to the samples and they were taken through a second heating step. The samples were then diluted to 10 ml with deionized water. The digested samples were analyzed for trace element concentrations by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Concentrations of As, Ba, Cd, Cr, Cu, Fe, Mn, Sr, Tl, V and Zn, (He collision mode), Se (reaction mode), and Hg (normal mode) were quantified in each sample. Digestion quality control measures included digestion blanks, fortified blanks, and reference materials at a frequency of 1 each per 20 samples. There was insufficient material to allow for digestion of duplicates or spikes. Analytical sample duplicates and spikes were performed at a frequency of 1 each per twenty samples. Additional quality control consisted of reporting limit checks, interference checks, and initial and continuing calibration checks and blanks.

Detection limits for each sample varied because the mass of each sample used in the analysis varied. If the trace element concentration was below the detection limit, we assigned that sample a concentration of half of the detection limit for statistical comparisons. For egg samples, the average detection limits were (ug/g dry mass) As 0.015 µg/g, Ba 0.048 µg/g, Cd 0.004 µg/g, Cr 0.223 µg/g, Cu 0.143 µg/g, Fe 7.423 µg/g, Mn 0.069 µg/g, Hg 0.074 µg/g, Se 0.053 µg/g, Sr 0.022 µg/g, Tl 0.004 µg/g, V 0.029 µg/g, and Zn 1.061 µg/g. In egg samples, As, Cd, Cr, and V concentrations were below the detection limit in over half of the samples from each colony and were not considered further. The average detection limits for nestling blood samples (µg/g wet mass) for each element were As 0.009 µg/g, Ba 0.049 µg/g, Cd 0.009 µg/g, Cr 0.098 µg/g, Cu 0.080 µg/g, Fe 8.224 µg/g, Mn 0.068 µg/g, Hg 0.029 µg/g, Se 0.312 µg/g, Sr 0.020 µg/g, Tl 0.009 µg/g, V 0.016 µg/g, and Zn 2.17 µg/g. In blood samples, As, Cd, Cr, Mn, Tl, V concentrations were below the detection limit in over half of the samples from each colony and were not considered further. Average relative % difference for eight trace elements over five analysis duplicates was 12 ± 2%. Average % recovery for 13 trace elements over five analysis spiked samples was 97 ± 21%. Average % recovery for Mn, Fe, Cu, Zn, As, Se, Sr, Cd, and Hg was 100 ± 13% for five separate digestions of NIST 2976. Chromium recovery averaged 48%, presumably because the Cr was in a form that is not solubilized by the open vessel acid digestion used here. Other elements were not certified in the NIST standard.

ANALYSIS

We used Kolmogorov-Smirnov tests and normality plots to determine if variables met the assumptions of parametric tests. Any variables that did not meet parametric assumptions were transformed to improve normality or analyzed using an appropriate nonparametric test. We primarily used ANCOVAs with colony as a random factor, nest as the unit of replication, and clutch initiation date and/or brood size as covariates. In all ANCOVAs, we examined the interaction between location and the covariate to ensure that the assumption of slope homogeneity was met. If the covariate did not contribute significantly to the model ($p > 0.10$) then it was removed from the model to improve statistical power. When significant differences among sites were detected, we used Tukey post-hoc tests to determine where those differences occurred.

We compared nest box use, recapture rates, and the effects of the cold snap between colonies using a Chi-square test. We compared clutch initiation dates among colonies using an ANOVA. To examine the effects of trace element exposure on egg size, we calculated egg volume using the formula Length*Width^2*0.51 (Hoyt, 1979). We then compared egg mass and volume between colonies using ANOVA. We calculated hatching success as the proportion of eggs that hatched in the nest of those that remained after we collected fresh eggs for trace element analysis. We only examined hatching success in nests where at least one egg hatched and that were not affected by the 2011 cold snap. We did this to exclude nests that failed completely due to other weather events, predation, or disturbance caused by researchers or other human activities that were unrelated to the ash spill. We calculated fledging success as the proportion of young present in the nest at day 17 out of the number of eggs that were laid after adjusting for our collection of fresh eggs. We arcsine square root transformed these proportion data prior to analysis and compared reproductive success among colonies using an ANCOVA and initially included the number of young present and nest initiation dates as covariates. We used the residuals from the regression of nestling body mass on average tarsus length as a measure of body condition (mass controlling for structural size), which a study in European Starlings indicated was a good index of lipid reserves and hence condition (Ardia, 2005). To examine the effects of colony location on nestling size and condition, we used a nested ANCOVA where nestlings were nested within their nest box to avoid pseudoreplication. We initially included clutch initiation date and number of young present as covariates in these analyses but dropped them from final models because they were not significant.

We log transformed trace element values and used principal components analysis to produce variables that were not correlated with each other (Table 2). We used a MANOVA to compare egg and blood principal components (PC) scores among colonies to determine if exposure to contaminants was higher in areas associated with the spill. To examine the effects of trace element exposure on these reproductive parameters, we performed backward stepwise regressions using the PCs as independent variables. We used PCs generated from egg trace element concentrations to examine the effect of contaminant exposure on egg size, clutch size, hatching success, and clutch initiation date. We used PCs produced from nestling blood trace element concentrations to examine the effects of contaminants on nestling size, condition, and fledging success. At the time this annual report was finalized (March, 2013), trace element data were not yet available for the 2012 field season so we limit our discussion of trace elements to 2011 data. All statistical tests were two-tailed and alpha = 0.05. All analyses were performed with PASW 18 (SPSS).

RESULTS

COLONY UTILIZATION, RECAPTURE RATE, WEATHER EFFECT AND PREDATION

In 2011, the overall proportion of nest boxes in which tree swallows initiated clutches (i.e., nest box occupancy) was 77.0% (361/469), but occupancy differed significantly among colonies (χ^2 = 25.40, df = 8, p = 0.001). Over 85% of nest boxes at Downstream 2 (30/32), Downstream 3 (39/43), and the Spill Site (80/94) had clutches initiated in them by Tree Swallows. Nest box occupancy at Reference 3 (34/53), Downstream 1 (22/32), and Downstream 4 (32/51) was substantially lower with fewer than 69 % of nest boxes occupied by Tree Swallows at these colonies. Settlement at Melton Hill Dam (52/68), Reference 1 (34/46), and Reference 2 (38/50) was intermediate with around 75% of boxes occupied by Tree Swallows in these areas. In 2012, nest box occupancy increased to 86.6% (406/469), and occupancy again differed significantly among colonies (χ^2 = 36.849, df = 8, p < 0.001). Over 90% of the nest boxes at Reference 1 (34/35), the Spill Site (81/85), Downstream 1 (31/34), and

Downstream 2 (30/32) had clutches initiated in them by Tree Swallows. Reference 3 (50/58), Downstream 3 (40/46), and Melton Hill Dam (54/61) had clutches initiated in 86-88% of the nest boxes. Downstream 4 (39/60) had clutches initiated in only 65% of the boxes and Reference 2 (47/58) had Tree Swallow clutches in 81 % of the boxes.

We banded 1544 adult and nestling Tree Swallows in 2011 and 2513 in 2012. Of the birds captured in 2012, 277 were recaptured adults (43.0 % of banded adults) and 66 were recaptured nestlings (7.5 % of banded nestlings) from 2011. We examined the effects of nest success, sex, and colony on recapture rates for birds that were banded as adults in 2011. We categorized the nests of adults as successful if at least one of the young they produced with their social mate survived to day 17. For males, this excludes extra-pair young that may have been produced with neighboring females. Female recapture rate was affected by their 2011 nest success; females that abandoned or whose nests were depredated were recaptured at a significantly lower rate (27.4%; 34/124) in 2012 than females who bred successfully at the site the prior year (59.2 %; 126/213, χ^2 = 31.65, df = 1, p < 0.001). Male recaptures were not affected by nest success with his social mate (χ^2 = 0.058, df = 1, p = 0.810); individuals who were successful breeders returned at similar rates as those who were unsuccessful (47.6 %; 80/168 and 50.0%; 15/30, respectively). Because we found sex-specific difference in the response to nest failure, we compared recapture rates between sexes in two ways. First, we included all birds regardless of their breeding success and found that recapture rate did not differ between males and females (χ^2 = 0.489, df = 1, p = 0.484). Second, we excluded adults whose nests were unsuccessful from further analyses in order to focus on other environmental effects (i.e., trace element exposure) and found that a greater proportion of females (59.2%; 126/213) than males (47.6 %; 80/168) were recaptured in 2012 (χ^2 = 5.03, df = 1, p = 0.025). Female recapture rate did not differ significantly among colonies (Figure 2, χ^2 = 7.90, df = 8, p = 0.444) but male recapture rate did (Figure 2, χ^2 = 16.44, df = 8, p = 0.036). Males that bred at Reference 1 and Downstream 1 and 4 were less likely to be recaptured than males at other colonies while males from Downstream 2 were more likely to be recaptured. We also compared recapture rate between female age classes and found no difference in the proportion of each age class recaptured (χ^2 = 0.892, df = 1, p = 0.345, SY female: 63.5 %; 47/74, ASY female: 56.8%; 79/139).

We examined recapture rates of birds that were banded as nestlings in 2011 and found that 55 different nests produced young that returned in 2012. Typically, a single nestling returned but seven nests recruited two young and a single nest produced three returning young. Young from nests with early clutch initiation dates were more likely to return to the breeding population than those with later clutch initiation dates (Wald = 10.15, df = 1, p = 0.001), but brood size did not affect the likelihood of recapture (Wald = 0.000, df = 1, p = 0.998). Clutch initiation date did not differ significantly among colonies in 2011 ($F_{8, 220}$ = 1.428, p = 0.186) so we did not include this variable in analyses that compared nestling return rate among colonies. The number of broods that recruited young into the breeding population did not differ significantly among colonies (Table 2, χ^2 = 10.93, df = 8, p = 0.206) nor did the number of young recruited from each colony (Table 2, Kruskall Wallis Test, χ^2 = 11.19, df = 8, p = 0.191).

Between May 15th and May 19th 2011, a period of unseasonably cold weather occurred and caused nestling mortality or complete nest failure that affected nests in most of the colonies. We found significant differences among colonies in the proportion of broods that lost at least one nestling or failed to hatch eggs following the cold snap (Figure 3, χ^2 = 16.465, df = 8, p = 0.036). A greater proportion of nests lost young or experienced complete nest failure at the Spill Site (10/55), Melton Hill Dam (11/37), and Reference 1 (7/27) with 18 – 29 % of nests affected in these colonies. In contrast, the other colonies all suffered less than 11% nestling and egg mortality that was attributable to the cold snap (Reference 3 = 2/29, Reference 2 = 3/30, Downstream 1 = 0/18, Downstream 2 = 2/21, Downstream 3 = 3/31, Downstream 4 = 3/28).

In 2011, during the first week of June, a raccoon depredated 47 of the 71 active nests at the Spill Site. Of the depredated nest boxes, only 5 pairs subsequently had re-nesting activity that resulted in the production of nestlings. We also detected signs of raccoon predation (muddy paw prints on predator guards) at Downstream 4 in 2011, though fewer Tree Swallows were present at this colony making the predation less destructive. In 2012, predator guards were modified and raccoons were trapped at the Spill Site, Reference 1, Reference 2, Reference 3, and Downstream 4. None of the colonies experienced raccoon nest predation in 2012. We also compared predation rates among colonies in both years. In 2011, 47% (205/448) of nests were depredated and predation rates differed significantly among colonies (Figure 4, χ^2 = 17.617, df = 8, p = 0.024). At the Spill Site, Downstream 1 and 4, over 50% of the nests that were initiated were depredated. In 2012, only 16% (75/465) of the nests that were initiated were depredated and predation rates differed significantly among colonies (Figure 4, χ^2 = 34.968, df = 8, p < 0.001). Predation rates at the Spill Site and Reference 1 were under 7% but predation rates at Downstream 1 and 4 remained over 32%.

REPRODUCTIVE SUCCESS AMONG TREE SWALLOW COLONIES

We compared reproductive success among colonies with reproductive data from 2011 and 2012 combined. We found that nest initiation date ($F_{8, 1085}$ = 1.159, p = 0.321), clutch size (χ^2 =12.516, df = 8, p = 0.130), egg volume ($F_{8, 690}$ = 0.575, p = 0.799), and egg mass ($F_{8, 684}$ = 0. 530, p = 0.834) were similar among colonies (Figure 5). Hatching success did not differ among colonies (Figure 6, $F_{8, 694}$ = 0.951, p = 0.473) but fledging success did (Figure 6, $F_{8, 575}$ = 2.290, p = 0.020). Post-hoc tests revealed that Downstream 1 had significantly higher fledging success than Melton Hill Dam (p = 0.017) and tended to be greater than at Downstream 2 (p = 0.059).

We found that aspects of nestling quality differed among colonies (Figure 7). Nestling mass differed significantly among colonies ($F_{8, 718}$ = 6.209, p < 0.001) and post-hoc tests revealed that nestlings from Reference 2 and Melton Hill Dam were significantly smaller than nestlings from all of the other colonies (all p $\leq$ 0.002) except for Downstream 3. Nestlings from Downstream 4 weighed significantly more than nestlings from all other colonies (all p $\leq$ 0.024). Nestlings from Reference 3 were also significantly heavier than nestlings from all other colonies (all p < 0.016) except Downstream 2 (p = 0.277) and Downstream 4. Wing length also differed significantly among colonies ($F_{8, 702}$ = 4.835, p < 0.001). Nestlings from Reference 2 and Melton Hill Dam had significantly shorter wings than nestlings from the Spill Site, Reference 3, Downstream 1, and Downstream 4 (all p $\leq$ 0.016). Nestlings from Downstream 4 and Reference 3 had significantly longer wings than nestlings from all other colonies (all p $\leq$ 0.049). Average tarsus length also differed significantly among colonies ($F_{8, 757}$ = 2.202, p < 0.025). Nestlings at Melton Hill Dam had significantly shorter tarsi than nestlings at the Spill Site, Downstream 1, 2 and 3, and Reference 3 (all p $\leq$ 0.013). Nestlings from Reference 3 had significantly longer tarsi than nestlings from the Spill Site (p = 0.041) and Downstream 3 (p = 0.017) as well. Nestling condition also differed significantly among colonies ($F_{8, 732}$ = 6.105, p < 0.001) and nestlings from Reference 2 were in significantly worse condition and nestlings from Downstream 4 in significantly better condition than nestlings from all other colonies (all p $\leq$ 0.001). Nestlings from Melton Hill Dam were in significantly worse condition than those from the Spill Site and Reference 1 and 2 as well (all p $\leq$ 0.001).

MATERNAL TRANSFER OF TRACE ELEMENTS AND EMBRYONIC EXPOSURE AMONG COLONIES

We examined maternal transfer of trace elements to eggs by determining if there were relationships between female blood trace element concentrations and those found in eggs (Figure 8). Maternal blood concentrations seven

days prior to or following oviposition were significantly positively correlated with egg concentrations of Fe ($r^2 = 0.112$, p = 0.035), Hg ($r^2 = 0.651$, p < 0.001) and Se ($r^2 = 0.102$, p = 0.044), and we detected a positive trend for Sr concentrations ($r^2 = 0.082$, p = 0.073). Maternal blood and egg concentrations of Ba ($r^2 = 0.026$, p = 40), Cu ($r^2 = 0.052$, p = 0.157), and Zn ($r^2 = 0.008$, p = 0.594) were not significantly related.

We also examined maternal transfer and embryonic exposure by examining trace element concentrations found in all of the fresh eggs collected from the colonies. Principal components analysis produced three principal components that together explained 60.9% of the variance in egg trace element concentrations (Table 3). PC1 received high, positive factor loadings for Hg, Se, Sr, and Tl and explained 27.7% of the variance. PC2 received high, positive factor loadings for Ba and Zn while PC3 received high factor loadings for Fe, Mn, and a negative loading for Cu. PC2 explained 19.4% and PC3 explained 13.9% of the variance in trace element concentrations. Exposure to contaminants associated with PC1 differed significantly among colonies and post-hoc tests revealed that eggs from the Spill Site had significantly higher PC1 scores than the other colonies (Figure 9, all p < 0.001). All of the Downstream colonies had significantly higher egg PC1 scores than all of the reference colonies (all $p \leq 0.031$) and Downstream 1 had significantly higher scores than Melton Hill Dam as well (p < 0.001). Egg PC2 ($F_{8, 258} = 0.938$, p = 0.486) and PC3 ($F_{8, 258} = 1.432$, p = 0.183) scores did not differ significantly among colonies. Embryonic exposure near the site of the fly-ash spill was greater for many maternally transferred trace elements associated with PC1 (Table 4).

We examined the effect of trace element exposure on clutch size and egg size in Tree Swallows. We found that clutch size was not significantly correlated with PC1 ($R_s = 0.010$, n = 267, p = 0.870) or PC2 ($R_s = 0.082$, n = 267, p = 0.180) but that there was a significant positive relationship with PC3 (Figure 10, $R_s = 0.209$, n = 267, $p \leq 0.001$) indicating that eggs with high Fe, Mn and low Cu concentrations were in larger clutches. We found that egg volume ($F_{1, 265} = 1.332$, $R^2 = 0.001$, p = 0.249), egg mass ($F_{1, 265} = 1.736$, $R^2 = 0.003$, p = 0.189), and hatching success ($F_{1, 231} = 0.631$, $R^2 = -0.002$, p = 0.428) were unrelated to contaminant exposure.

BLOOD TRACE ELEMENT CONCENTRATIONS AND NESTLING SIZE AND CONDITION

Principal components analysis produced three principal components of blood trace element contaminant concentrations in nestlings (Table 3). PC1 explained 23.8% of the variance in contaminant concentrations and received high, positive factor loadings for Cu, Hg, and Zn and a moderate loading for Se and Fe. PC2 received moderate factor loadings for Ba and Se while PC3 received a high factor loading for Sr and a moderate one for Ba. PC2 explained an additional 17.6% of the variance and PC3 explained 16.1% of the variance. Contaminant exposure varied among colonies (Table 5) and PC1 scores differed significantly among colonies (Figure 11, $F_{8, 224} = 8.970$, p < 0.001). Nestlings at the Spill Site had significantly higher PC1 scores than all of the other colonies with the exception of Melton Hill Dam, Reference 2, and Downstream 1 (all $p \geq 0.093$). Melton Hill Dam had significantly higher PC1 scores than Downstream 3, Reference 1, and Reference 3 (all $p \leq 0.028$). Scores for PC2 also differed significantly among colonies ($F_{8, 224} = 3.189$, p = 0.002) but in this case nestlings at Melton Hill Dam had significantly higher scores than those at Reference 1, Reference 3, and Downstream 3 (all $p \leq 0.034$) and there was a trend for them to be higher than at Downstream 1 (p = 0.081) and the Spill Site (p = 0.092). PC3 scores did not differ significantly among colonies ($F_{8, 224} = 1.232$, p = 0.281).

We examined the effects of the three principal components of blood trace element exposure on nestling size and condition. We found a weak negative relationship between wing length and PC3 scores (Figure 12, $F_{1, 234} = 7.894$, p = 0.005, $R^2 = 0.033$). Nestling body mass ($F_{1, 234} = 2.008$, p = 0.158, $r^2 = 0.004$), tarsus length ($F_{1, 233} = 2.181$, p = 0.141, $R^2 = 0.005$), and condition ($F_{1, 233} = 1.108$, p = 0.294, $R^2 = 0.001$) were not related to any of the

trace element principal components. All three principal components were also unrelated to the proportion of the brood that survived to day seventeen ($F_{1, 174}$ = 1.200, p = 0.275, R^2 = 0.083).

DISCUSSION

The 2008 Kingston coal-fly ash spill released a large quantity of coal-fly ash slurry into the Emory River that then flowed into the Clinch and Tennessee Rivers (TVA, 2009). In the 2.5 years since the spill, extensive dredging of the Emory River removed most of the fly-ash from the system. However, it is important to determine if remediation efforts have been successful. Thus, we examined Tree Swallow reproductive success, aspects of nestling quality, and recapture rates in areas impacted by the spill and at nearby reference colonies.

In 2011, we detected lower nest box usage at Reference 3 and at Downstream 1 and 4 compared to other colonies (Table 6). It is possible that lower occupancy at two of these sites in 2011 was attributable to nest box placement; we altered the location and number of nest boxes at Reference 3 appreciably in 2011 and we established the Downstream 1 colony in 2011. Tree swallows often settle more quickly in areas where they have bred previously or that they identify in the late summer prior to migration (Adams and Brewer, 1981; Lombardo, 1987). Thus, it was not surprising that in 2012 settlement rates at Reference 3 and Downstream 1 were comparable to settlement rates at other colonies. The lower settlement at Downstream 4 in 2011 persisted into 2012 and is more difficult to interpret as that colony has been established for several years. Low settlement at this colony could be due to competition from other secondary cavity nesters in the area. For example, numerous Eastern Bluebirds and some Carolina Chickadees and Prothonotary Warblers occupied nest boxes at Downstream 4 at higher frequencies (23.4 %) than other colonies (5.2-14.0 %).

We examined recapture rates of adults and nestlings that were banded in 2011. For birds banded as adults in 2011, we found that female recapture rate was affected by their breeding success while male recapture rate was not. Females may be more sensitive to reproductive failure and prospect for a new breeding site if a breeding attempt is unsuccessful (Hoover, 2003). Males may be less likely to do this because they have the opportunity to sire offspring in neighboring nests through extra-pair copulations and so may not experience complete reproductive failure if their nest fails (Hoover, 2003). Moreover, extra-pair copulations are very common in Tree Swallows and it is likely that males did not sire all of the offspring in their nest box, further reducing the value of that reproductive attempt (Whittingham and Dunn, 2001). Because nest failure affected males to a lesser extent than females, we compared recapture rates between the sexes two ways, including failed nests and excluding them. When we included failed nests, we found no difference between the sexes in their recapture rate. However, when we excluded nests that failed, we found that the recapture rate of females was higher than that of males and that male but not female recapture rate differed among colonies (Table 6). Males were less likely to be recaptured if they bred at Reference 1 or Downstream 1 and 4. We captured females more easily and earlier in the nesting cycle than males and this likely contributed to the difference in recapture rate, particularly at Downstream 1 and 4 where predation rates were high. Studies on other swallow species have detected higher annual survival in females than in males (Brown and Brown, 1996; Møller and Szép, 2002) but the mechanism underlying this difference is unclear. Without complete trace element data, it is impossible to determine if trace element exposure affects male or female recapture rate.

We found that nestlings from broods initiated earlier in the season were more likely to return to the breeding population the following year, but that brood size did not affect nestling return rate. A number of studies have also found greater recruitment from nests initiated earlier in the season and this is often attributed to declining resource

availability later in the season (Müller et al., 2005; Tarof et al., 2011; Verboven and Visser, 1998; Wheelwright et al., 2003). Other studies have found that young from larger broods are less likely to recruit into the breeding population (Magrath, 1991; Morton et al., 2004; Newton, 1989; Tarof et al., 2011) but that was not the case in our study. Our collection of an egg for trace element analysis reduced brood size, and in turn reduced within-brood competition for resources provided by parents, and thus may have inhibited our ability to detect an effect of brood size on nestling recapture rate. We did not find any significant differences among colonies in nestling recapture rate though an additional year of recapture data is necessary to produce more conclusive results.

The recapture data presented here should not be construed as an estimate of survival. Data sets used to examine survival include hundreds or even thousands of recapture or re-sighting events over several years (Custer et al., 2012; Stutchbury et al., 2009). The maximum likelihood models most frequently use to estimate survival probability incorporate estimates of detection probability, the chance an individual survived to the next sampling event but was not detected (White and Burnham, 1999). Detection probability cannot be estimated with a single year of recapture data and may differ among sexes, age classes, or colonies and being unable to include it can bias estimates of survival probability. For instance, female recapture rate in our study was higher than that of males but this was likely because their detection probability was higher (i.e., they were easier to capture), not their survival. The data we gathered in 2011 and 2012 was an excellent starting point for a study examining the effects of trace elements on Tree Swallow survival. However, construction of a park at the Spill Site during the 2013 breeding season made it impossible for us to quantify return rates and detection probability for this colony, leaving survival rates among colonies unanswered.

Similar to other studies, we found a potentially interactive effect of trace element exposure and inclement weather on reproductive success in Tree Swallows. Birds nesting at the Spill Site, an impacted colony, and Melton Hill Dam, a positive control, suffered the greatest nestling mortality and hatching failure that corresponded with a late season cold-snap. Our result is similar to those of other studies that found that severe weather events exacerbated the effects of pollutants on reproductive success (Eeva and Lehikninen, 2010; Hallinger and Cristol, 2011). However, we found that pairs breeding at Reference 1 also suffered similar high rates of nest failure during the cold-snap. Reference 1 may be subject to anthropogenic disturbance (due to a nearby marina, dam, and highway) that could exacerbate the effects of inclement weather on reproductive success in Tree Swallows.

We examined maternal transfer of contaminants to eggs by first focusing on the relationship between female blood and egg trace element concentrations. We found that maternal blood concentrations correlated well with egg concentrations for Hg and weakly for Fe, Se, and Sr. Ecotoxicology studies of avian species typically examine embryonic exposure by collecting one or more eggs from each clutch (Custer et al., 2010). However, this practice reduces clutch and brood sizes and that could affect hatching success, nestling growth, and parental investment (de Heij et al., 2006; Partridge and Harvey, 1985; Wheelwright et al., 1991). Contaminant exposure often produces greater reproductive effects when combined with additional environmental stressors, such as poor weather conditions (Hallinger and Cristol, 2011), and reducing brood size likely leads to less demanding rearing conditions for nestlings and adults. It would be ideal to use female blood concentrations in lieu of collecting eggs. Based on our results, it may be possible to use maternal blood Hg concentrations collected within 7 days of oviposition to predict egg concentrations in Tree Swallows. Another study in Tree Swallows also found a tight relationship between maternal blood Hg and egg Hg concentrations (Brasso et al., 2010). For the other elements, the relationship between maternal blood and egg concentrations was too weak to warrant using maternal blood to estimate embryonic exposure.

Female Tree Swallows near the spill maternally transferred several trace elements to eggs at concentrations elevated above reference levels. Females at the Spill Site and Downstream 1 had significantly higher PC1 scores

and thus had eggs that contained more Se, Sr, and Tl than other colonies. Despite greater contaminant exposure at these colonies, we found no significant differences among colonies in nest initiation date, clutch size, egg size, or hatching success. We did find that fledging success was greater at Downstream 1 than at one of the reference colonies and Melton Hill Dam. With the 2011 trace element data, we found no effect of trace element concentrations on these aspects of reproductive success including fledging success. The lack of reproductive effects suggests that Tree Swallows are likely not exposed to contaminant concentrations that could cause reproductive impairment. Selenium is one of the primary drivers of ecological risk in systems impacted by fly-ash and egg hatchability is the most sensitive indicator of Se toxicity in avian species (Ohlendorf, 2003). Egg Se concentrations of 10 mg/kg dry weight are the predicted lower threshold for detecting reproductive effects, though species vary in their sensitivity to Se (Heinz, 1996; Janz et al., 2010). The highest egg Se concentrations we detected were at the Spill Site and Downstream 1 and were slightly more than 3 mg/kg dry mass (range 2.34 – 5.19 mg/kg), below concentrations that affect hatching success and in most cases similar to those necessary for normal development in poultry (Puls, 1994). In another study on Tree Swallows, nestling blood Hg concentrations of 0.23 ug/g were associated with reduced fledging success, but this result was potentially affected by maternal transfer of Hg to eggs, egg size, and female breeding experience (Brasso and Cristol, 2008). The highest blood Hg concentrations that we detected were found at Reference 2 and were only 0.03 ug/g, concentrations similar to those found at reference colonies in the other study (Brasso and Cristol, 2008). Another study on Tree Swallows found no effect of trace element exposure on hatching and fledging success and had similar egg concentrations of Ba, Sr, Fe, Zn, and Mn as those found in this study (Custer et al., 2006).

Studies in other species have found effects of trace elements and metals on egg size in birds and those concentrations were typically higher than those found in this study (Nyholm and Myhrberg, 1977). However, another study that examined the effects of Hg on Tree Swallow reproduction found no effect of very high Hg levels on egg or clutch size (Brasso and Cristol, 2008; Hallinger and Cristol, 2011). It is possible that egg and clutch size are not influenced strongly by environmental factors in Tree Swallows. In another study on Tree Swallows, egg size was not influenced by many common measures of female quality such as body size and body mass (Whittingham et al., 2007). Some variation in egg mass is attributable to insect abundance 1-3 days prior to egg laying (Ardia et al., 2006; Whittingham et al., 2007) but egg size seems to be a highly heritable trait in Tree Swallows (Wiggins, 1990). Thus, it may be difficult to detect and/or interpret effects of trace element exposure on egg size in Tree Swallows due to other environmental and genetic effects.

Nestling exposure to trace elements differed among colonies. Nestlings at the Spill Site, Downstream 1, and Melton Hill Dam had significantly higher scores for nestling blood PC1 and PC2 and were exposed to more Se, Sr and Ba than nestlings in other colonies. However, when we looked at the effects of trace element exposure on nestling size and condition, we only detected one weak, negative relationship between exposure to PC3 and wing length. This could mean that exposure to Sr and Ba negatively affected feather growth in nestling Tree Swallows. Other studies have indicated that high Se concentrations can reduce growth and condition in avian young (Heinz et al., 1987; Ohlendorf, 2003) but a study of shorebirds and terns detected no effect of liver Se concentrations on nestling condition (Ackerman and Eagles-Smith, 2009). Studies in other passerines exposed to similar contaminants have detected effects of trace element exposure on nestling condition and body mass (Eeva and Lehikninen, 1996; Janssens et al., 2003), but concentrations of contaminants were higher in those studies than in our study.

We also found nestling size and condition differed among the colonies but these differences may not be related to trace element contamination (Table 6). Nestlings at Reference 2 and at Melton Hill Dam, the positive control colony, were in many cases smaller and in worse condition than nestlings at other colonies. We also found that nestlings from Downstream 4 and Reference 3 were often larger and in better condition than those in other

colonies. The differences in nestling quality among colonies may be due to exposure to low concentrations of contaminants, ecological differences among colonies, exposure to other sources of anthropogenic disturbance, interactions between these factors, or merely natural variation in nestling quality. Reduced nestling quality at Melton Hill Dam may be related to exposure to trace element contaminants in this section of the Clinch River as well as elevated human recreational activity at this colony. Reference 2 also experiences high levels of human disturbance, which could inhibit incubation or provisioning of young for multiple days and cause the reduction in nestling quality we detected at this colony. Size and body condition at fledging strongly influence survival and recruitment in other species (Gebbhardt-Henrich and Richner, 1998; Tinbergen and Boerlijst, 1990) and young from these colonies may be less likely to recruit into the breeding population.

Overall, our initial results suggest that residual trace element exposure following remediation of the 2008 coal-fly ash spill has little effect on Tree Swallows in the Kingston area. However, it is difficult to draw robust conclusions with a single year of trace element data. The 2011 trace element concentrations in the impacted colonies and at Melton Hill Dam ("positive control") were typically below levels that cause reproductive effects in avian species. With the 2012 data, we will be able to determine if trace element exposure is declining in this system and if the lack of direct reproductive effects are consistent from year to year. Our results suggest that simultaneous exposure to multiple stressful conditions (trace elements plus human activity or inclement weather), could impair Tree Swallow reproductive success and lead to the differences in fledging success and nestling quality we detected (Table 6). Our future work will focus on evaluating more subtle, physiological endpoints such as the immune and stress responses once we obtain the 2012 trace element data.

REFERENCES

ACAA, 2011. 2011 Coal Combustion Product (CCP) Production and Use Survey Report. Farmington Hills, MI: American Coal Ash Association. p. 1.

Ackerman JT, Eagles-Smith CA, 2009. Selenium bioaccumulation and body condition in shorebirds and terns breeding in San Francisco Bay, California, USA. Environ Toxicol Chem 28:2134-2141.

Adams RJJ, Brewer R, 1981. Autumn selection of breeding location by Field Sparrows. The Auk 98:629-630.

ARCADIS. Draft Trace Element Concentrations in Tree Swallows: 2009-2010. Kingston: TVA; 2011. TVA.

Ardia DR, 2005. Super size me: an experimental test of the factors affecting lipid content and the ability of residual body mass to predict lipid stores in nestling European Starlings. Functional Ecology 19:414-420. doi: 10.1111/j.1365-2435.2005.00997.x.

Ardia DR, Wasson MF, Winkler DW, 2006. Individual quality and food availability determine yolk and egg mass and egg composition in tree swallows Tachycineta bicolor. Journal of Avian Biology 37:252-259. doi: 10.1111/j.2006.0908-8857.03624.x.

Beck ML, Hopkins WA, Jackson BP, In review. Spatial and temporal variation in the diet of tree swallows: Implications for trace element exposure following habitat remediation. Arch Environ Contam Toxicol.

Brasso RL, Abdel Latif MK, Cristol DA, 2010. Relationship between laying sequence and mercury concentration in Tree Swallow eggs. Environ Toxicol Chem 29:1155-1159.

Brasso RL, Cristol DA, 2008. Effects of mercury exposure on the reproductive success of tree swallows (*Tachycineta bicolor*). Ecotoxicology 17:133-141. doi: 10.1007/s10646-007-0163-z.

Brown CR, Brown MB, 1996. Coloniality in the Cliff Swallow: The Effect of Group Size on Social Behavior Chicago: University of Chicago Press.

Bryan AL, Hopkins WA, Baionno JE, Jackson BP, 2003. Maternal transfer of contaminants to eggs of Common Grackles (Quiscalus quiscala) nesting on coal fly ash basins. Arch Environ Contam Toxicol 45:273-277.

Burger JR, Gochfield M, 1997. Risk, mercury levels, and birds: relating adverse laboratory effects to field biomonitoring. Environmental Research 75:160-172.

Cook RB, Suter GW, Sain ER, 1999. Ecological risk assessment in a large river-reservoir: 1. Introduction and background. Environ Toxicol Chem 18:581-588. doi: 10.1897/1551-5028(1999)018<0581:eraial>2.3.co;2.

Custer CM, 2011. Swallows as sentinel species for contaminant exposure and effect studies. In: JE E, CA M, CA B, editors. Wildlife Ecotoxicology: Forensic Approaches New York: Springer. p. 45-91.

Custer CM, Custer TW, Hines JE, 2012. Adult tree swallow survival on the polychlorinated biphenyl-contaminated Hudson River, New York, USA, between 2006 and 2010. Environ Toxicol Chem 31:1788-1792. doi: 10.1002/etc.1894.

Custer CM, Custer TW, Warburton D, Hoffman DJ, Bickham JW, Matson CW, 2006. Trace element concentrations and bioindicator response in tree swallows from northwestern Minnesota. Environ Monit Assess 118:247-266.

Custer CM, Gray BR, Custer TW, 2010. Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallows, *Tachycineta bicolor*. Environ Toxicol Chem 29:909-921.

de Heij ME, van den Hout PJ, Tinbergen JM, 2006. Fitness cost of incubation in great tits (Parus major) is related to clutch size. Proceedings of the Royal Society B-Biological Sciences 273:2353-2361. doi: 10.1098/rspb.2006.3584.

Dunn PO, Hannon SJ, 1992. Effects of food abundance and male parental care on reproductive success and monogamy in tree swallows. Auk 109:488-499.

Eeva T, Lehikninen E, 1995. Egg shell quality, clutch size, and hatching success of the great tit (*Parus major*) and the pied flycatcher (*Ficedula hypoleuca*) in an air pollution gradient. Oecologia 102:312-323.

Eeva T, Lehikninen E, 1996. Growth and mortality of nestling great tits (*Parus major*) and pied flycatchers (*Ficedula hypoleuca*) in a heavy metal pollution gradient. Oecologia 108:631-639.

Eeva T, Lehikninen E, 2010. Polluted environment and cold weather induce laying gaps in great tit and pied flycatcher. Oecologia 162:533-539.

Epstein PR, Buonocore JJ, Eckerle K, Hendryx M, Stout III BM, Heinberg R, Clapp RW, May B, Reinhart NL, Ahern MM, Doshi SK, Glustrom L, 2011. Full cost accounting for the life cycle of coal. In: Costanza R, Limburg K, Kubiszewski I, editors. Ecological Economics Reviews New York: Annals of the New York Academy of Sciences. p. 73-98.

Gebbhardt-Henrich S, Richner H, 1998. Causes of growth variation and its consequences for fitenss. In: Starck JM, Ricklefs RE, editors. Avian Growth and Development: Evolution within the Altricial-Precocial spectrum Oxford: Oxford University Press. p. 324-339.

Hallinger KK, Cristol DA, 2011. The role of weather in mediating the effect of mercury exposure on reproductive success in tree swallows. Ecotoxicology 20:1368-1377. doi: 10.1007/s10646-011-0694-1.

Harding LE, 2008. Non-linear uptake and hormesis effects of selenium in red-winged blackbirds (*Agelaius phoeniceus*). Sci Total Environ 389:350-366.

Heinz GH, 1996. Selenium in birds. In: Beyer WN, Heinz GH, Redmon-Norwood AW, editors. Environmental contaminants in wildlife: interpreting tissue concentrations Boca Raton, Fl: CRC Lewis. p. 447-458.

Heinz GH, Hoffman DJ, 2003. Predicting mercury in mallard ducklings from mercury in chorioallantoic membranes. Bulletin of Environmental Contamination and Toxicology 70:1242-1246.

Heinz GH, Hoffman DJ, Krynitsky AJ, Weller DMG, 1987. Reproduction in mallards fed selenium. Environ Toxicol Chem 6:423-433.

Hoover JP, 2003. Decision rules for site fidelity in a migratory bird, the Prothonotary Warbler. Ecology 84:416-430.

Hoyt DF, 1979. Practical methods of estimating volume and fresh weight of bird eggs. The Auk 96:73-77.
IEA, 2012. Key World Energy Statistics. Paris, France: International Energy Agency.

Janssens E, Dauwe T, Pinxten R, Bervoets L, Blust R, Eens M, 2003. Effects of heavy metal exposure on the condition and health of nestlings of the great tit (*Parus major*), a small songbird species. Environ Pollut 126:267-274.

Janz DM, DeForest DK, Brooks ML, Chapman PM, Gilron G, Hoff D, Hopkins WA, McIntyre DO, Mebane CA, Palace VP, Skorupa JP, Wayland M, 2010. Selenium toxicity to aquatic organisms. In: Chapman PM, Adams WJ, Brooks ML, Delos CG, Luoma SN, Maher WA, Ohlendorf HM, Presser TS, Shaw DP, editors. Ecological Assessment of Selenium in the Aquatic Environment New York, New York: CRC Press. p. 141-231.

Lombardo MP, 1987. Attendants at tree swallow nests. II. The exploratory-dispersal hypothesis. . Condor 89:138-149.

Magrath RD, 1991. Nestling Weight and Juvenile Survival in the Blackbird, Turdus merula. J Anim Ecol 60:335-351. doi: 10.2307/5464.

Møller AP, Szép T, 2002. Survival Rate of Adult Barn Swallows Hirundo rustica in Relation to Sexual Selection and Reproduction. Ecology 83:2220-2228. doi: 10.2307/3072053.

Morton ML, Pereyra ME, Crandall JD, MacDougall-Shackleton EA, Hahn TP, 2004. Reproductive Effort and Return Rates in the Mountain White-Crowned Sparrow The Condor 106:131-138. doi: 10.1650/7304.

Müller M, Pasinelli G, Schiegg K, Spaar R, Jenni L, 2005. Ecological and social effects on reproduction and local recruitment in the red-backed shrike. Oecologia 143:37-50. doi: 10.1007/s00442-004-1770-5.

Newton I, editor 1989. Lifetime reproduction in birds. London: Academic Press Limited.

NRC. Managing coal combustion residues in mines. Washington, D.C.: National Academies Press; 2006. National Academies Press.

Nyholm NEI, Myhrberg HE, 1977. Severe eggshell defects and impaired reproductive capacity in smal passerines in Swedish Lapland. Oikos 29:336-341.

Ohlendorf HM, 2003. Ecotoxicology of selenium. In: Hoffman DJ, Rattner BA, Burton GAJ, Cairns JJ, editors. Handbook of Ecotoxicology, 2nd ed Boca Raton, Fl: CRC.

Ohlendorf HM, 2011. Selenium, salty water, and deformed birds. In: Eliott JE, Morrissey CA, Bishop CA, editors. Wildlife Ecotoxicology: Forensic Approaches New York: Springer.

Partridge L, Harvey PH, 1985. Costs of reproduction. Nature 316:20.

Puls R, 1994. Mineral Levels in Animal Health, 2nd ed. Clearbrook, BC Canada: Sherpa International.

Quinney TE, Ankney CD, 1985. Prey size selection by tree swallows. Auk 102:245-250.

Robertson RJ, Stutchbury BJ, Cohen RR, Winkler DW, Hallinger KK, Ardia DR, 2011. Tree Swallow. In: Poole A, editor. The Birds of North America-On-Line, revised ed Ithaca: Cornell Lab of Ornithology.

Rowe CL, Hopkins WA, Congdon JD, 2002. Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: A review. Environ Monit Assess 80:207-276.

Stantec. TVA disposal facility assessment phase 1 plant summary Bull Run Fossil Plant (BRF). Nashville, TN: Stantec; 2009. Stantec, p. 76.

Stutchbury BJ, Robertson RJ, 1987. Two methods of sexing adult tree swallows before they begin breeding. Journal of Field Ornithology 58:236-242.

Stutchbury BJM, Hill JR, Kramer PM, Rush SA, Tarof SA, 2009. Sex and age-specific annual survival in a neotropical migratory songbird, the Purple Martin (*Progne subis*) Auk 126:278-287. doi: 10.1525/auk.2009.08038.

Tarof SA, Kramer PM, Hill JR, Tautin J, Stutchbury BJM, 2011. Brood size and late breeding are negatively related to juvenile survival in a neotropical migratory songbird Auk 128:716-725. doi: 10.1525/auk.2011.11087.

Tinbergen JM, Boerlijst MC, 1990. Nestling weight and survival of individual great tits (*Parus major*). J Anim Ecol 59:1113-1127.

TVA, 2009. Corrective Action Plan for the TVA Kingston Fossil Plant Ash Release. Kingston.

TVA. Inspection report: TVA's groundwater monitoring at coal combustion products disposal areas. Knoxville, TN: Office of Inspector General, Tennessee Valley Authority; 2011a. Office of Inspector General, Tennessee Valley Authority, p. 29.

TVA. TVA Kingston Fossil Fuel Plant Release Site On-Scene Coordinator Report for the Time-Critical Removal Action May 11, 2009 through December 2010. Harriman, TN: TVA; 2011b. Report No.: EPA-AO-030. TVA, p. 222.

USDA. Lower Clinch River rapid watershed assessment. TN: USDA and NRCS; 2009. USDA and NRCS, p. 19.

USEIA, 2012. Coal.

Verboven N, Visser ME, 1998. Seasonal variation in local recruitment of Great Tits: The importance of being early. Oikos 81:511-524.

Weech SA, Scheuhammer AM, Wayland ME, 2012. Selenium accumulation and reproduction in birds breeding downstream of a uranium mill in northern Saskatchewan, Canada. Ecotoxicology 21:280-288.

Wheelwright NT, Leary J, Fitzgerald C, 1991. THE COSTS OF REPRODUCTION IN TREE SWALLOWS (TACHYCINETA-BICOLOR). Can J Zool 69:2540-2547. doi: 10.1139/z91-358.

Wheelwright NT, Tice KA, Freeman-Gallant CR, 2003. Post-fledging parental care in Savannah Sparrows: Sex, size, and survival. Animal Behaviour 65:435-443.

White GC, Burnham KP, 1999. Program MARK: survival estimation from populations of marked animals. Bird Study 46:120-139.

Whittingham LA, Dunn PO, 2001. Survival of extrapair and within-pair young in tree swallows. Behavioral Ecology 12:496-500.

Whittingham LA, Dunn PO, Lifjeld JT, 2007. Egg mass influences nestling quality in tree swallows, but there is no differential allocation in relation to laying order or sex. The Condor 109:585-594.

Wiggins DA, 1990. Food availability, growth, and heritability of body size in neslting tree swallows (*Tachycineta bicolor*). Can J Zool 68:1292-1296.

TABLES AND FIGURES

TABLE 1.

Description of Tree Swallow colonies located around Kingston, TN in 2011 and 2012.

Colony Name	Location
Reference 1	Ft. Loudoun Dam on the Tennessee River, ≈ 35 km east of Kingston
Reference 2	Tellico Dam on the Little Tennessee River, ≈ 35 km east of Kingston
Reference 3	Long Island on the Tennessee River, 5.5 km upstream from confluence with Clinch River
Melton Hill Dam	On Clinch River, ≈ 35 km east of Kingston, served as positive control
Spill Site	Site of ash spill, on Emory River
Downstream 1	Power Lines cut located near Kingston Fossil Plant, on Emory River at confluence with Clinch River
Downstream 2	Kingston Fossil Plant Discharge Area, on Clinch River, 1.5 km from confluence with Emory River
Downstream 3	Kingston City Park, on Clinch River, 3 km from confluence with Emory River
Downstream 4	Pastures located on Tennessee River, 2.5 km downstream from confluence with Clinch River

TABLE 2.

2012 recapture of nestlings banded in 2011 by colony. We classified nests as having recruited young into the study population if one nestling from that brood was captured as an adult in 2012. No significant differences were detected among colonies in the number of nests that recruited young or in the total number of recruits produced out of the total number of nests that were initiated in that colony.

Location	Number of nests initiated	Number of nests that recruited young	Total number of recruits
Reference 1	34	5	6
Reference 2	38	3	3
Reference 3	34	4	4
Melton Hill Dam	52	9	13
Spill Site	80	11	12
Downstream 1	22	5	6
Downstream 2	30	10	12
Downstream 3	39	5	5
Downstream 4	32	4	4

TABLE 3.

Principal components factor loadings for Tree Swallow egg and blood contaminant concentrations. We performed PCA separately for egg and blood samples and each produced 3 principal components that together explained 61.0% (egg) and 57.5% (blood) of the variance in contaminant concentrations.

	Egg			Blood		
	PC1	PC2	PC3	PC1	PC2	PC3
Ba	0.106	0.824	-0.354	0.339	0.575	0.543
Cu	-0.027	-0.288	-0.419	0.649	-0.217	-0.151
Fe	0.130	0.124	0.714	0.495	0.254	-0.445
Mn	-0.120	0.078	0.327	BDL	BDL	BDL
Hg	0.755	-0.182	-0.242	0.551	-0.077	-0.174
Se	0.795	-0.199	0.325	0.450	0.594	0.012
Sr	0.722	0.474	-0.271	0.335	-0.373	0.759
Tl	0.845	-0.079	0.142	BDL	BDL	BDL
Zn	-0.101	0.809	0.268	0.516	-0.542	-0.057
% Variance	27.7	19.4	13.9	23.8	17.6	16.1

TABLE 4.

Mean and standard error for egg trace element concentrations among Tree Swallow colonies. Concentrations of Al, Sb, As, Be, Bo, Cd, Cr, Ni, Ag, and V were also quantified but were BDL in over half of the samples from all of the colonies and were not considered further.

Element	R1	R2	R3	MD	SS	D1	D2	D3	D4
Ba	6.44 ± 0.84	5.17 ± 0.81	4.16 ± 0.92	6.70 ± 0.68	6.15 ± 0.52	6.64 ± 1.24	4.73 ± 0.80	5.02 ± 0.80	4.49 ± 1.19
Co	0.07±0.005	0.08±0.004	0.10±0.005	0.07±0.004	0.07±0.003	0.07±0.007	0.08±0.004	0.07±0.004	0.08±0.007
Cu	2.08 ± 0.08	2.35 ± 0.07	2.18 ± 0.08	2.30 ± 0.06	2.14 ± 0.05	2.06 ± 0.11	2.09 ± 0.07	2.09 ± 0.07	2.20 ± 0.11
Fe	99.8 ± 3.9	115.7 ± 3.8	91.6 ± 4.23	105.1 ± 3.1	105.9 ± 2.4	113.2 ± 5.7	103.4 ± 3.7	100.5 ± 3.7	102.4 ± 5.5
Pb	0.01±0.002	0.01±0.002	0.01±0.003	0.02±0.002	0.01±0.002	0.01±0.004	0.01±0.002	0.01±0.002	0.01±0.003
Mn	3.67 ± 0.31	4.81 ± 0.29	4.84 ± 0.33	5.49 ± 0.25	4.26 ± 0.19	5.46 ± 0.45	4.86 ± 0.29	4.68 ± 0.29	4.50 ± 0.43
Hg	0.10 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.16 ± 0.01	0.25 ± 0.01	0.16 ± 0.02	0.16 ± 0.01	0.20 ± 0.01	0.13 ± 0.02
Mo	0.10±0.006	0.09±0.006	0.09±0.006	0.10±0.005	0.12±0.004	0.11±0.009	0.11±0.006	0.09±0.006	0.10±0.008
Se	2.39 ± 0.09	2.57 ± 0.09	2.39 ± 0.10	2.78 ± 0.07	3.45 ± 0.06	3.16 ± 0.14	2.82 ± 0.09	2.62 ± 0.09	2.79 ± 0.13
Sr	3.49 ± 0.27	3.09 ± 0.26	2.91 ± 0.30	3.28 ± 0.22	5.73 ± 0.17	5.51 ± 0.40	3.83 ± 0.26	3.77 ± 0.26	3.67 ± 0.39
Tl	0.01±0.002	0.02±0.002	0.01±0.002	0.02±0.002	0.05±0.001	0.02±0.003	0.03±0.002	0.02±0.002	0.03±0.003
Zn	67.51±1.96	66.04±1.89	64.97±2.13	66.21±1.58	63.62±1.21	67.71±2.89	65.89±1.86	64.92±1.86	68.47±2.77

TABLE 5.

Mean and standard error for nestling blood trace element concentrations among Tree Swallow colonies. Concentrations of Al, Sb, As, Be, Bo, Cd, Cr, Mn, Ni, Ag, and V were also quantified but were BDL in over half of the samples from all of the colonies and were not considered further.

Element	R1	R2	R3	MD	SS	D1	D2	D3	D4
Ba	0.66 ± 0.07	0.79 ± 0.08	0.64 ± 0.07	0.85 ± 0.05	0.84 ± 0.05	0.72 ± 0.07	0.82 ± 0.07	0.65 ± 0.06	0.78 ± 0.11
Co	0.02±0.004	0.02±0.004	0.01±0.004	0.01±0.003	0.02±0.003	0.01±0.004	0.02±0.004	0.03±0.003	0.01±0.006
Cu	0.27 ± 0.02	0.31 ± 0.02	0.30 ± 0.02	0.31 ± 0.01	0.34 ± 0.01	0.31 ± 0.02	0.32 ± 0.01	0.28 ± 0.01	0.32 ± 0.02
Fe	349.9±16.4	412.0±17.8	350.9±16.4	359.8±11.3	363.8±10.4	377.0±15.6	341.8±14.7	306.3±13.9	414.6±23.2
Pb	0.03±0.005	0.01±0.005	0.01±0.005	0.01±0.003	0.02±0.003	0.02±0.004	0.02±0.004	0.01±0.004	0.01±0.007
Hg	0.01±0.003	0.03±0.004	0.01±0.003	0.02±0.002	0.02±0.002	0.01±0.003	0.02±0.003	0.01±0.003	0.02±0.005
Mo	0.03±0.002	0.03±0.002	0.03±0.002	0.03±0.001	0.03±0.001	0.02±0.002	0.03±0.002	0.02±0.002	0.03±0.003
Se	0.87±0.18	0.94±0.19	0.77±0.18	2.26±0.12	1.73±0.11	1.03±0.17	1.11±0.16	1.07±0.15	1.08±0.25
Sr	0.08±0.13	0.08±0.14	0.09±0.13	0.28±0.09	0.11±0.08	0.14±0.13	0.09±0.12	0.09±0.13	0.07±0.19
Zn	6.41±0.30	6.15±0.33	6.13±0.30	6.02±0.21	6.92±0.19	6.14±0.29	6.29±0.27	6.45±0.25	5.75±0.43

TABLE 6.

Summary of ecological and reproductive differences among Tree Swallow colonies. In addition to differing in trace element concentrations, the colonies utilized in this study differed in a number of other factors that could have contributed to the differences in reproductive success and recapture rates that we detected. Other disturbance includes other types of anthropogenic disturbance that occur at colonies such as frequent human activity near nest boxes that could impede parental care at times. Habitat differences refer to the level of lawn care that occurs near nest boxes and can vary within a colony. Data on insect consumption among colonies can be found in Chapter 2.

Metric	R1	R2	R3	MD	SS	D1	D2	D3	D4
Other Disturbance	Moderate	High	Low	Moderate	Low	Low	Moderate	High	Low
Habitat	Open field	Cut grass & Open field	Open field	Cut grass	Cut grass	Open field & Cut grass	Cut grass	City & Cut grass	Open field
Predation	Low				Low	High 2x			High 2x
Settlement	High	Low	Moderate	Moderate	High	High	High	Moderate	Low
Insect Consumption	Terrestrial	Mixed	NA	Aquatic	Aquatic	Terrestrial	Mixed	Terrestrial	Terrestrial
Female Recapture									
Male Recapture	Reduced					Reduced	High		
Nestling Recapture									
Nest Initiation Date									
Cold Weather	Reduced			Reduced	Reduced				
Clutch Size									
Egg Volume									
Egg Mass									
Hatching Success									
Fledging Success				Reduced		High	Reduced		
Nestling Mass		Reduced	High	Reduced					High
Nestling Tarsus			High	Reduced					
Nestling Wing		Reduced	High	Reduced					High
Nestling Condition		Reduced		Reduced					High

FIGURE 1.

Tree Swallow colonies located near Kingston, TN. The study area consisted of two highly impacted colonies located on the Emory River. One was located at the site of the spill (Spill Site, SS, N = 94) and the second at the confluence of the Clinch and Emory River (Downstream 1, D1, N = 31). Moderately impacted colonies were located on the Clinch River at Downstream 2 (D2, N = 31) and Downstream 3 (D3, N = 43) and a low impacted colony was located downstream on the Tennessee River (D4, N = 51). We used three reference colonies; two were located near Lenoir City, TN 30.5 km east of Kingston. Reference 1 (R1, N = 46) was located at Ft. Loudoun Dam on the Tennessee River and Reference 2 (R2, N = 53) at Tellico Dam on the Little Tennessee River. Reference 3 was located on Long Island (R3, N = 53) on the Tennessee River upstream from the confluence with the Clinch River. We also placed boxes at Melton Hill Dam (MD, N = 68) on the Clinch River which served a role analogous to a positive control. MD, R1, and R2 are not pictured here. River kilometers are given in each river.
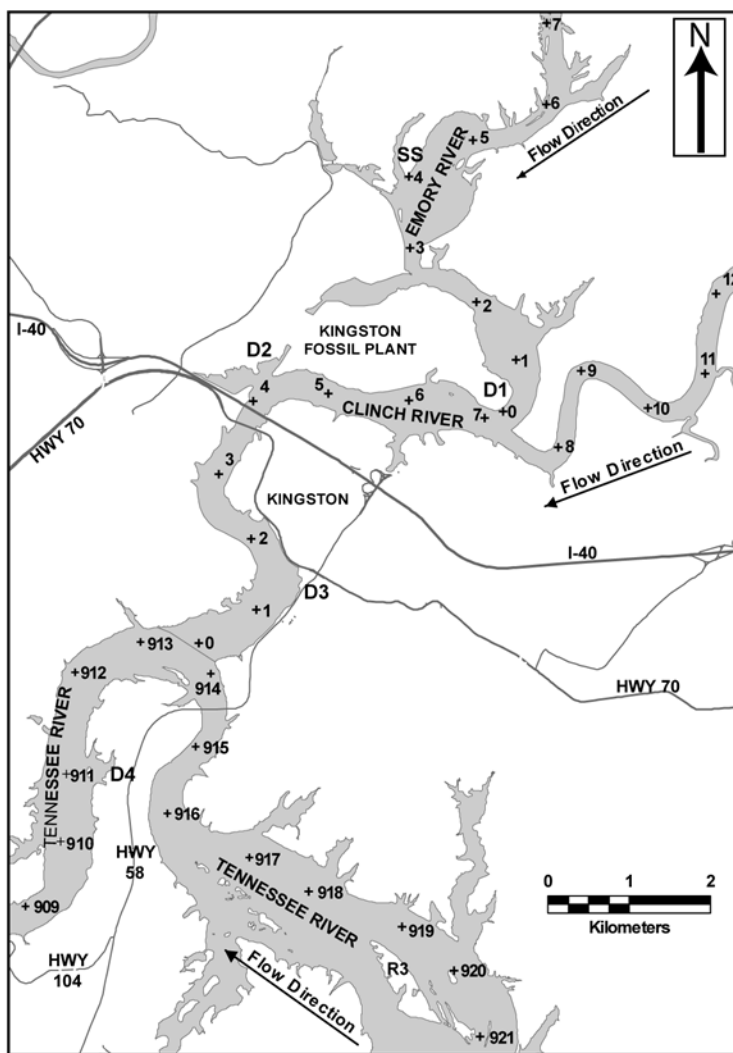
FIGURE 2.

The percentage of adult Tree Swallows banded as adults in 2011 and recaptured in 2012. We found that recapture rate of female Tree Swallows (open bars) was not significantly different among colonies but that the recapture rate for males (gray bars) was different. A smaller percentage of males that bred in Reference 1 and Downstream 1 and 4 in 2011 returned to one of the colonies in 2012. The recapture rate for males that bred in Downstream 2 in 2011 was higher than many of the colonies as well.

FIGURE 3.
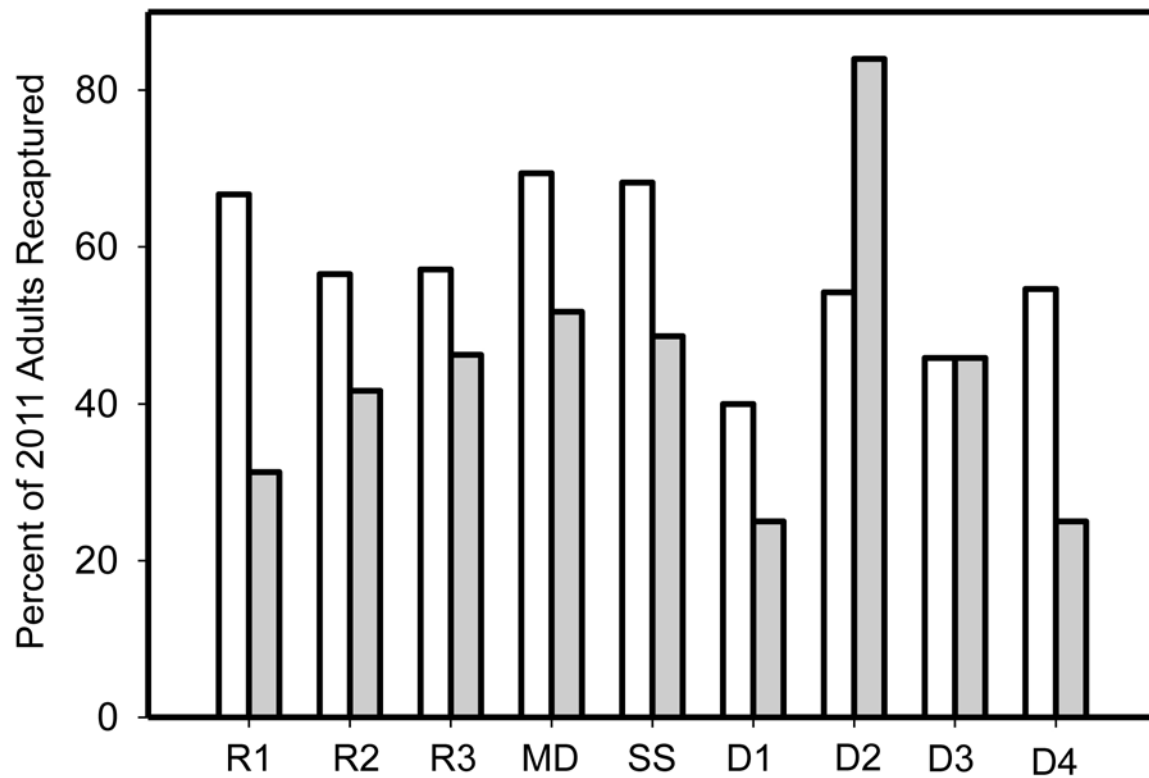
Nest failure among Tree Swallow colonies during unseasonably cold weather in 2011. Between May 15th and May 19th 2011, a period of unseasonably cold weather occurred and caused greater nestling mortality or complete nest failure the Spill Site, Melton Hill Dam, and Reference 1. In contrast, the other colonies all suffered less than 11% nestling and egg mortality during the cold snap.
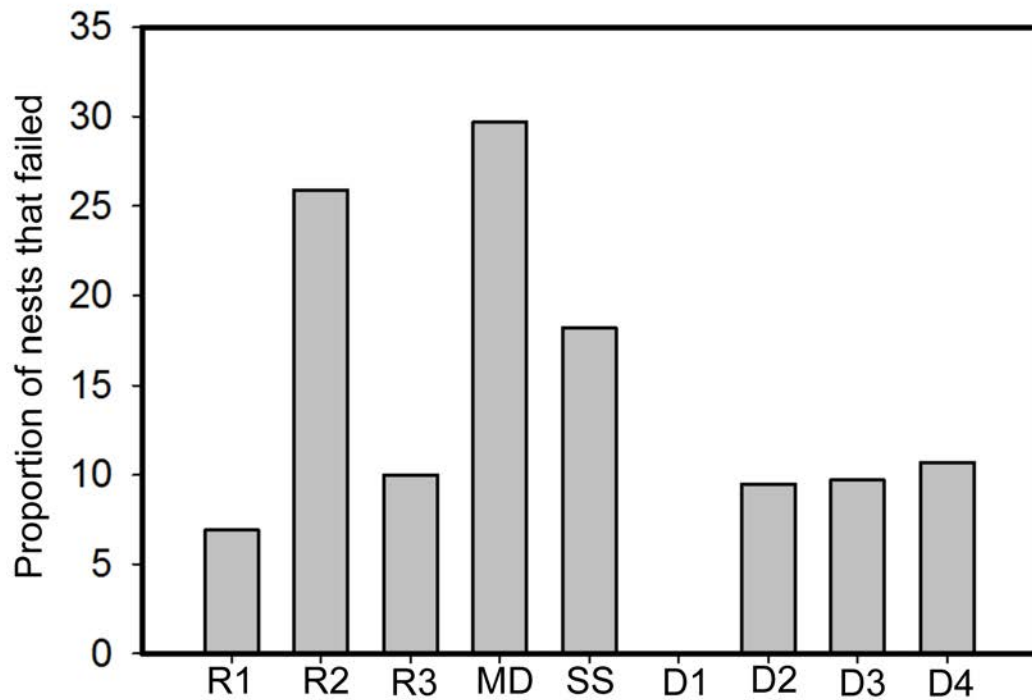
FIGURE 4.

Percentage of Tree Swallow nests depredated in 2011 and 2012 among colonies. In 2011, predation rates differed significantly among colonies (χ^2 = 17.617, df = 8, p = 0.024) and were highest at Downstream 1, the Spill Site, and Downstream 4. Some of the predation at the latter two colonies was attributable to raccoons that climbed the predator guards. In 2012 predation rates were lower but Downstream 1 and 4 still experienced greater predation than the other colonies while predation rates at the Spill Site and Reference 1 were quite low. White bars 2011, Gray bars 2012.
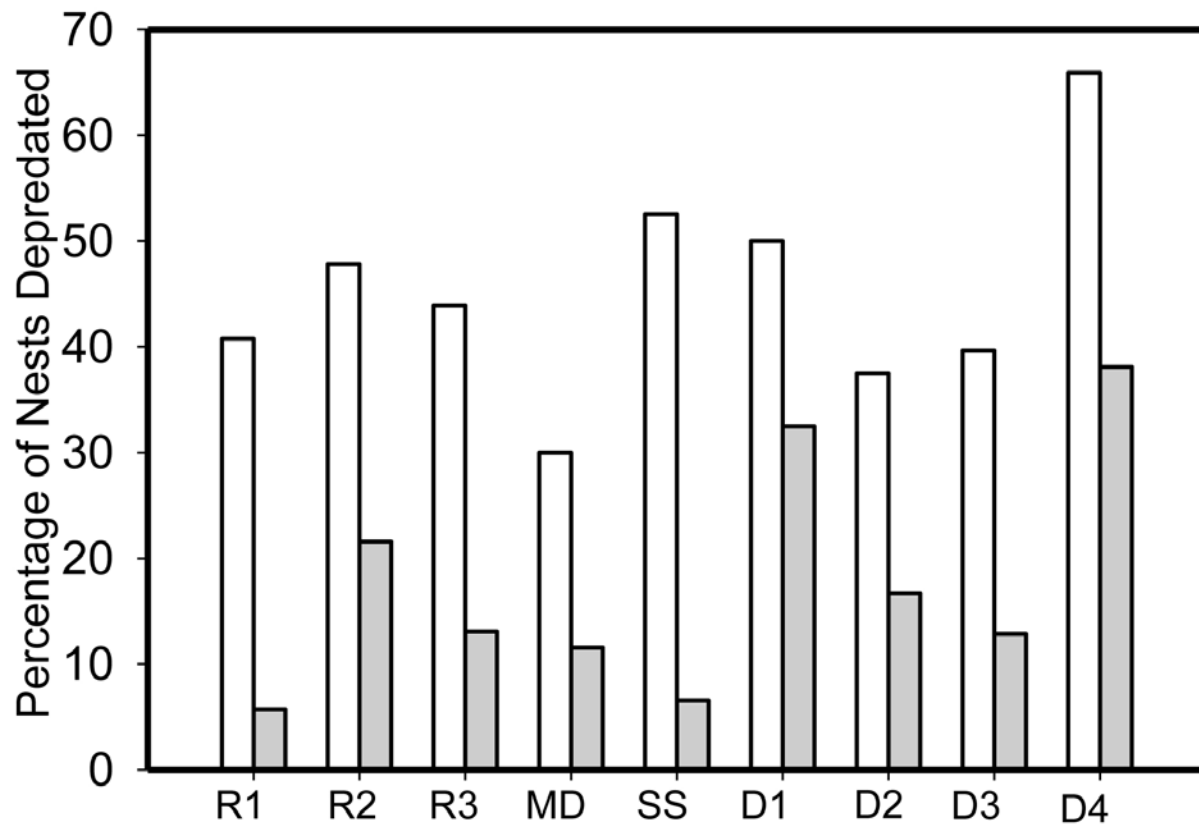
FIGURE 5.

Clutch initiation date and clutch investment among Tree swallow colonies. We combined reproductive data collected in 2011 and 2012 and found that clutch initiation date (a), clutch size (b), egg volume (c), and egg mass (d) did not differ significantly among Tree Swallow colonies. This suggests that trace element contaminants associated with the fly-ash spill are not affecting these aspects of Tree Swallow reproduction.
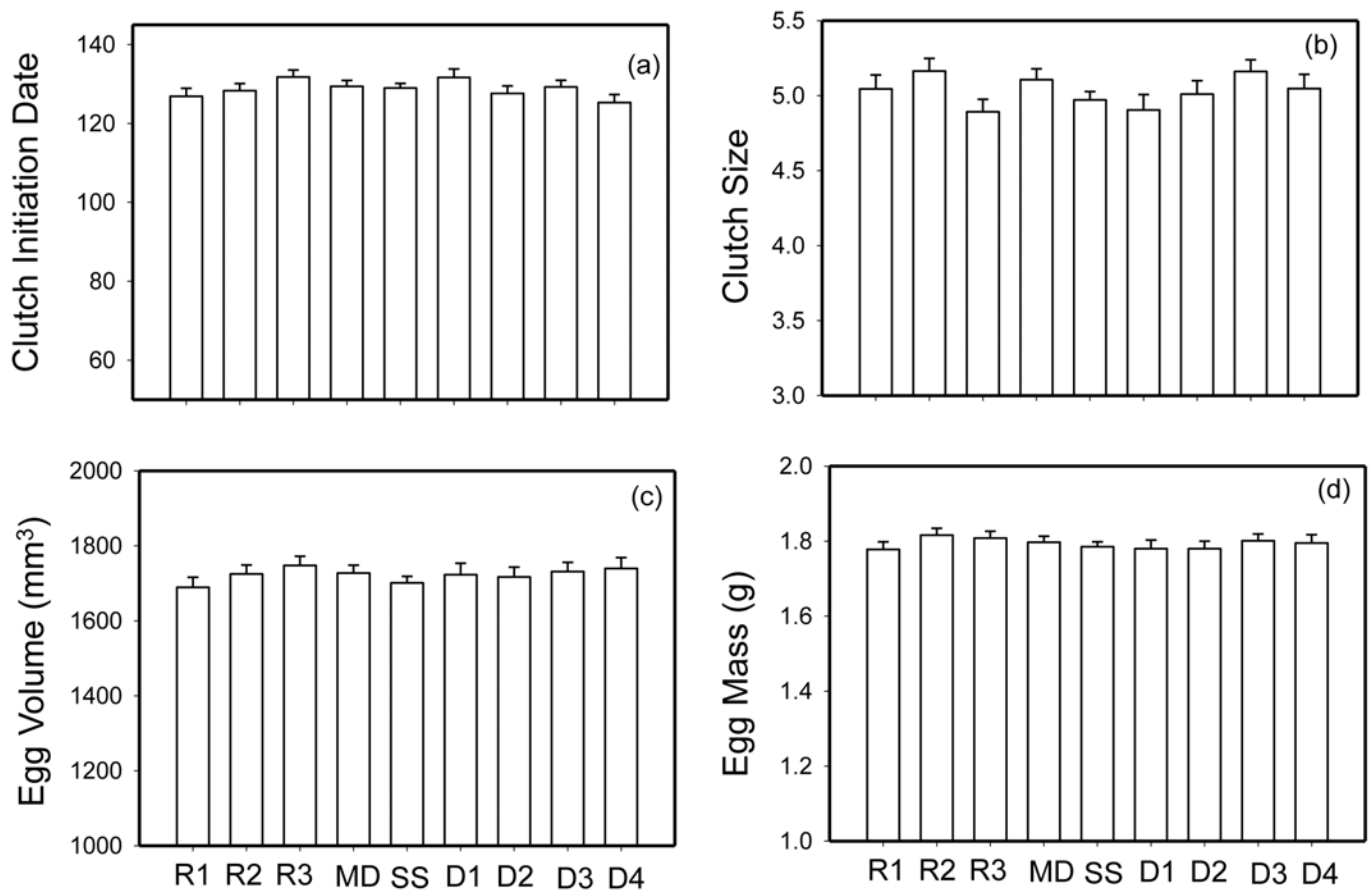
FIGURE 6.

Hatching (open bar) and fledgining (gray bar) success at Tree Swallow colonies in 2011 and 2012 following remediation of a coal-fly ash spill in Kingston, TN. We found that hatching success was similar among colonies in 2011 and 2012 but found reduced fledging success at Melton Hill Dam and Downstream 2 compared to Downstream 1. Fledging success at the Spill Site and Downstream 1 are similar to other colonies and so it seems that exposure to residual trace element contamination is not affecting either measure of reproductive success. Significant differences among colonies are denoted by different letters above bars.
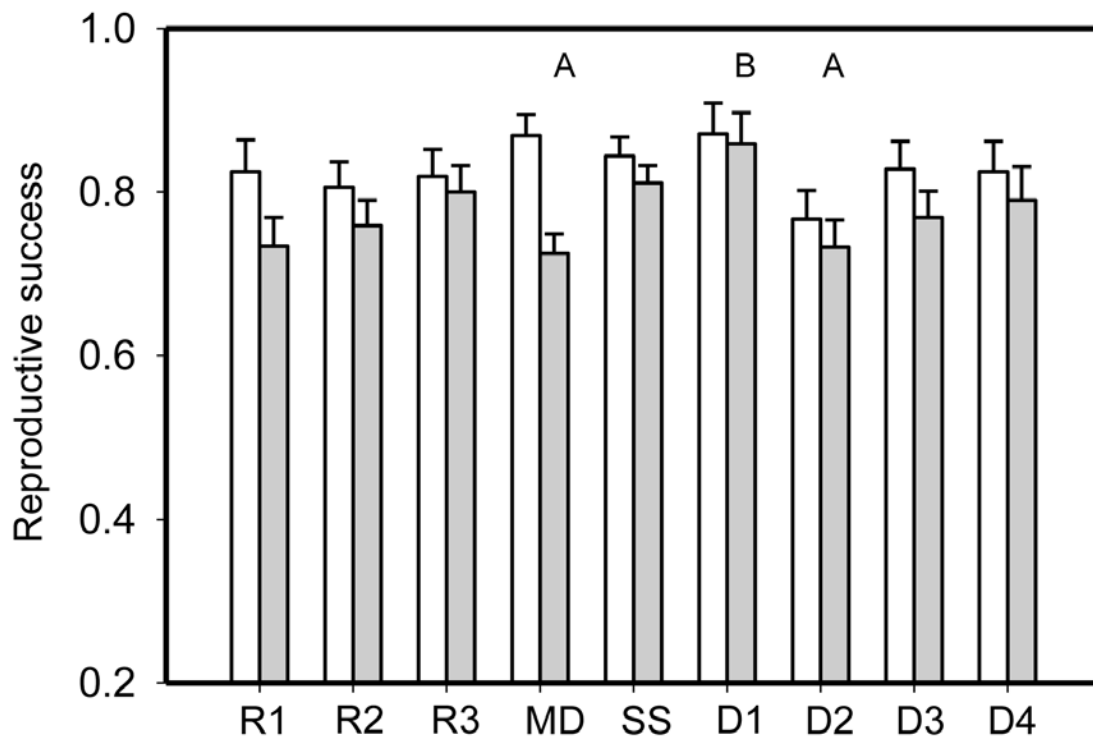
FIGURE 7.

Nestling size and condition among Tree swallow colonies. We combined reproductive data collected in 2011 and 2012 and found several significant differences among colonies in nestling body mass (a), wing length (b), tarsus length (c), and condition (d). Significant differences among colonies are indicated by different letters above bars. Generally, nestlings at Reference 2 and Melton Hill Dam were smaller than nestlings from the other colonies while those from Reference 3 and Downstream 4 were larger.
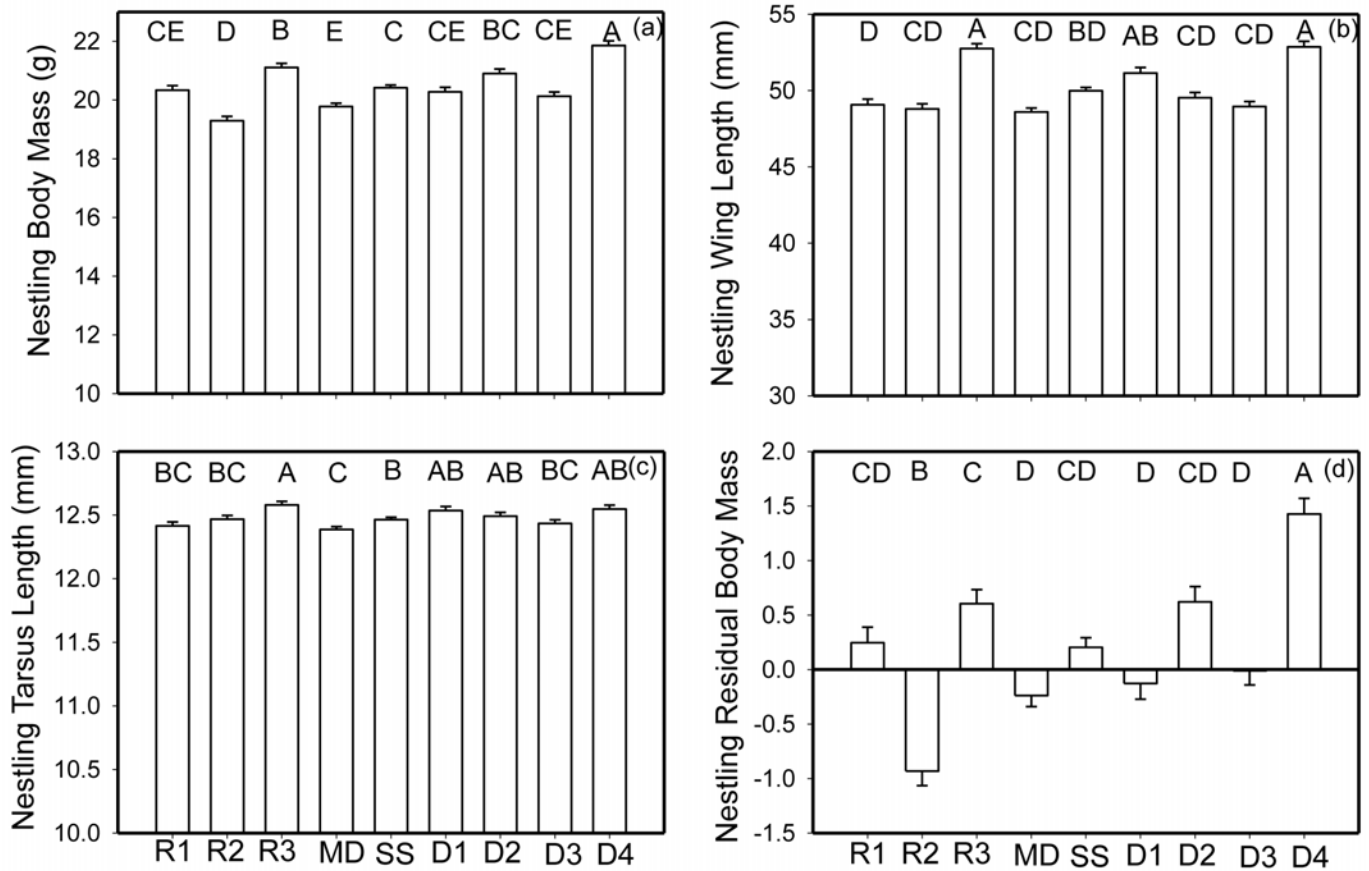
FIGURE 8.

Relationship between maternal blood trace element concentrations and egg trace element concentrations in Tree Swallows in 2011. Maternal blood concentrations were significantly positively correlated with egg concentrations of Fe (c), Hg (d), and Se (e) and we detected a trend for Sr (f) concentrations. Maternal blood and egg concentrations of Ba (a), Cu (b), and Zn (not pictured) were not significantly correlated. Analyses were performed on log transformed data but non-transformed data are shown in graphs for clarity.
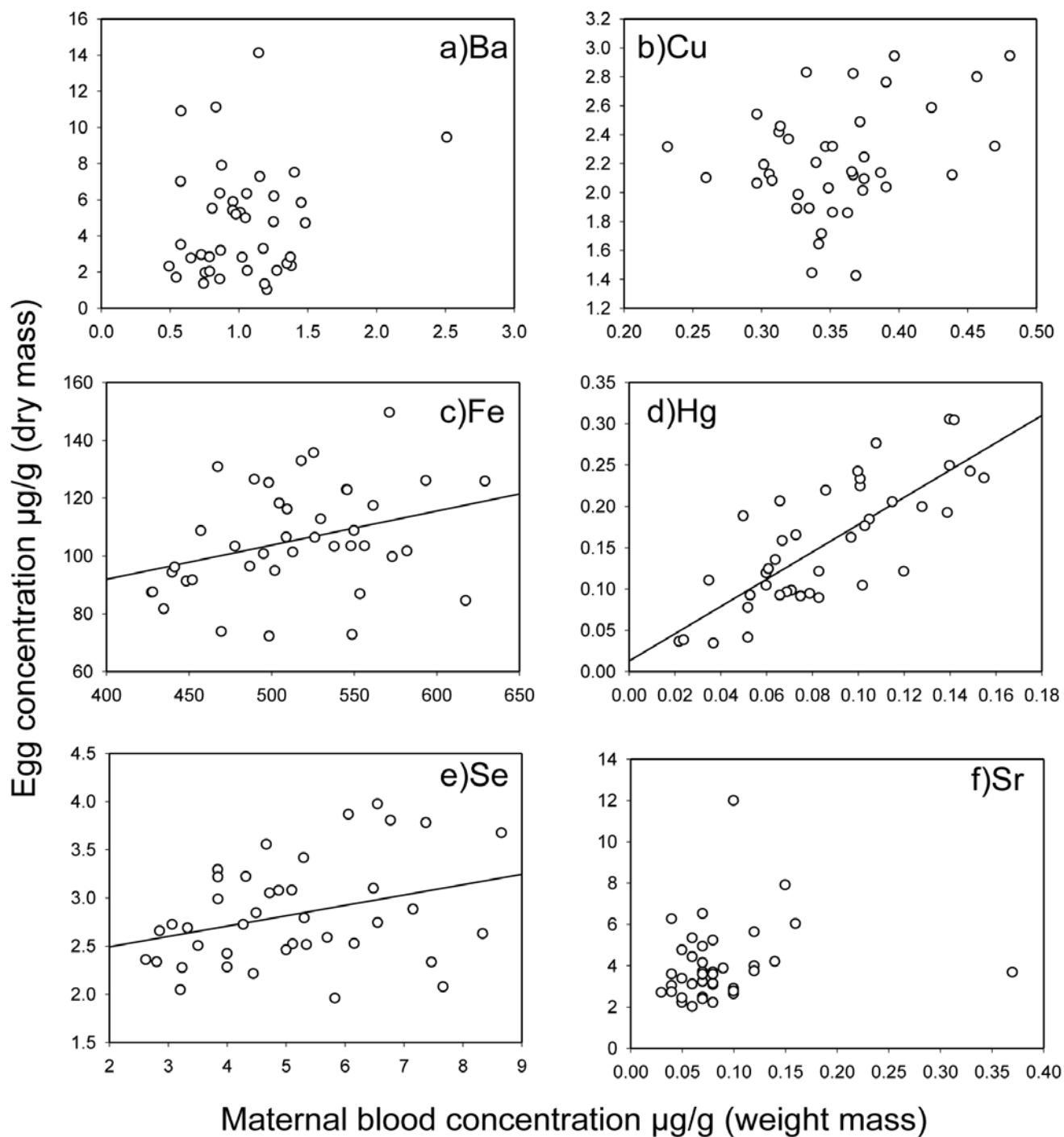
FIGURE 9.

Differences among Tree Swallow colonies in egg trace element exposure in 2011. Eggs at the Spill Site had significantly higher PC1 scores (gray bars) than all of the other colonies and PC1 scores were elevated at the downstream colonies compared to the reference colonies. Egg PC2 (black bars) and PC3 scores (open bars) did not differ significantly among colonies.
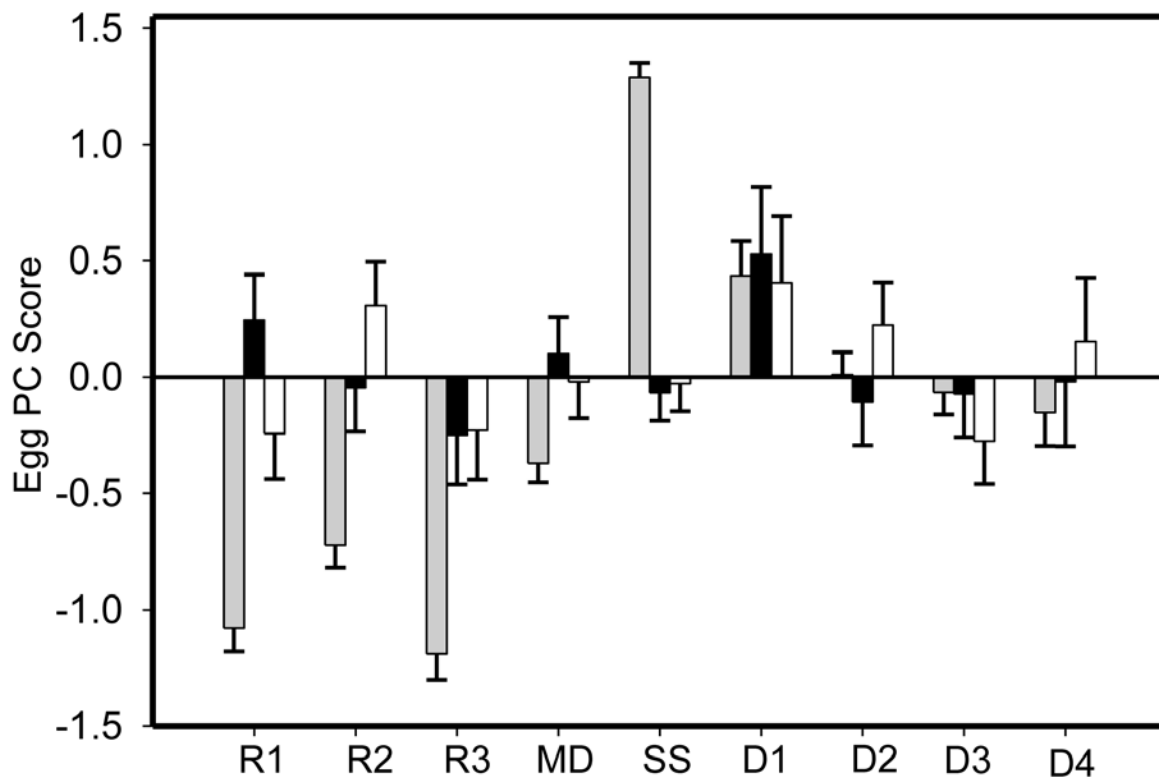
FIGURE 10.

Clutch size is positively correlated with exposure to contaminants associated with egg PC3. Egg PC3 received high, positive loadings for Fe and Mn and a strong negative loading for Cu. Iron and Mn would be necessary for normal embryonic development and this result may indicate that females able to lay larger clutches also allocated greater concentrations of resources necessary for embryonic development.
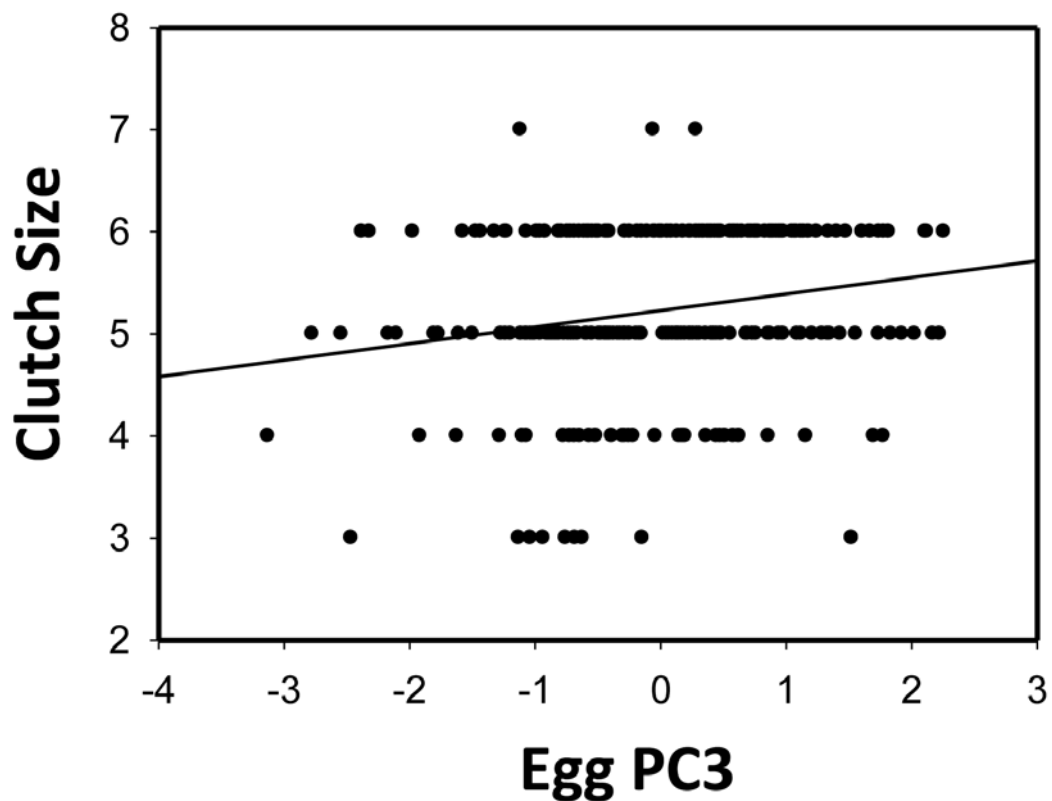
FIGURE 11.

Differences among Tree Swallow colonies in nestling blood trace element exposure in 2011. PC1 scores (gray bars) differed significantly among colonies and nestlings at the Spill Site had significantly higher PC1 scores than all of the other colonies with the exception of Melton Hill Dam, Reference 2, and Downstream 1. Melton Hill Dam had significantly higher PC1 scores than Downstream 3, Reference 1, and Reference 3. Scores for PC2 (black bar) also differed significantly among colonies but in this case nestlings at Melton Hill Dam had significantly higher scores than those at Reference 1, Reference 3, and Downstream 3. PC3 scores (open bars) did not differ significantly among colonies.
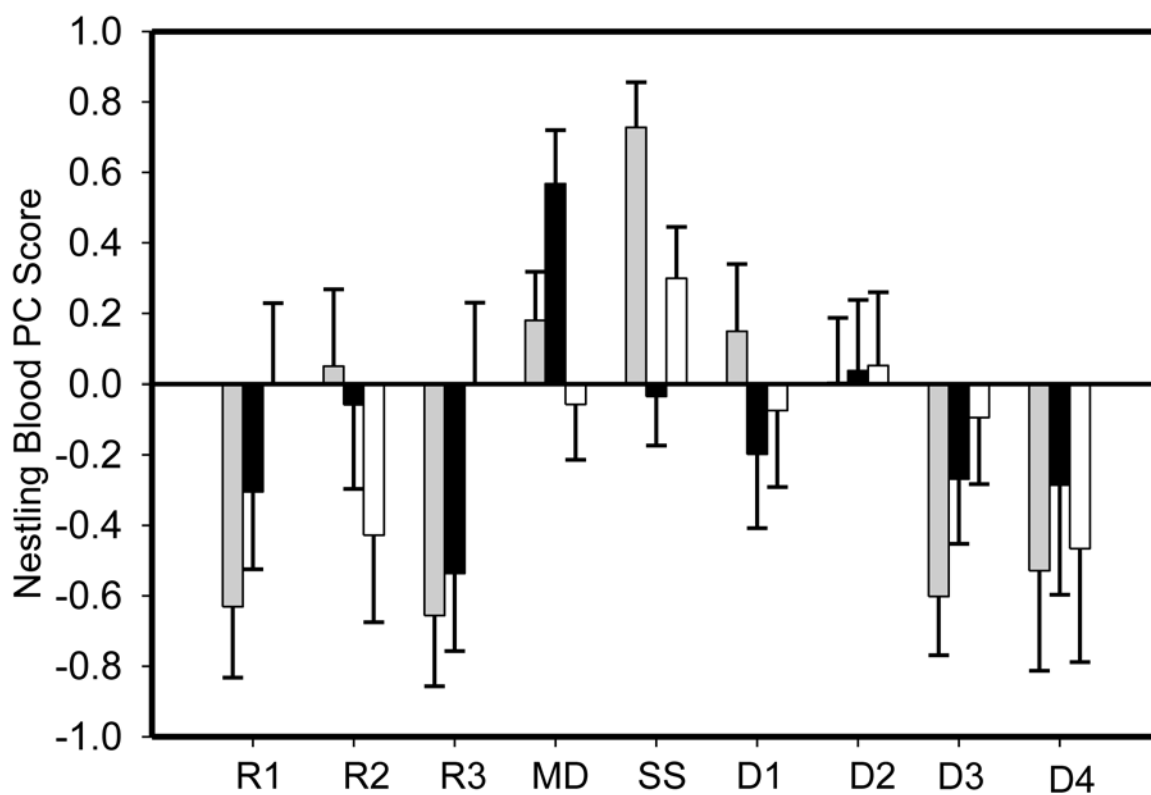
FIGURE 12.

Relationship between PC3 scores and wing length in neslting Tree Swallows in 2011. We found a statistically significant but weak relationship between nestling wing length and PC3 scores. PC3 received high factor loadings for Sr and Ba and nestlings with high scores had shorter wings. This may be indicative of a negative effect of these trace elements on nestling feather development though the relationship is weak.
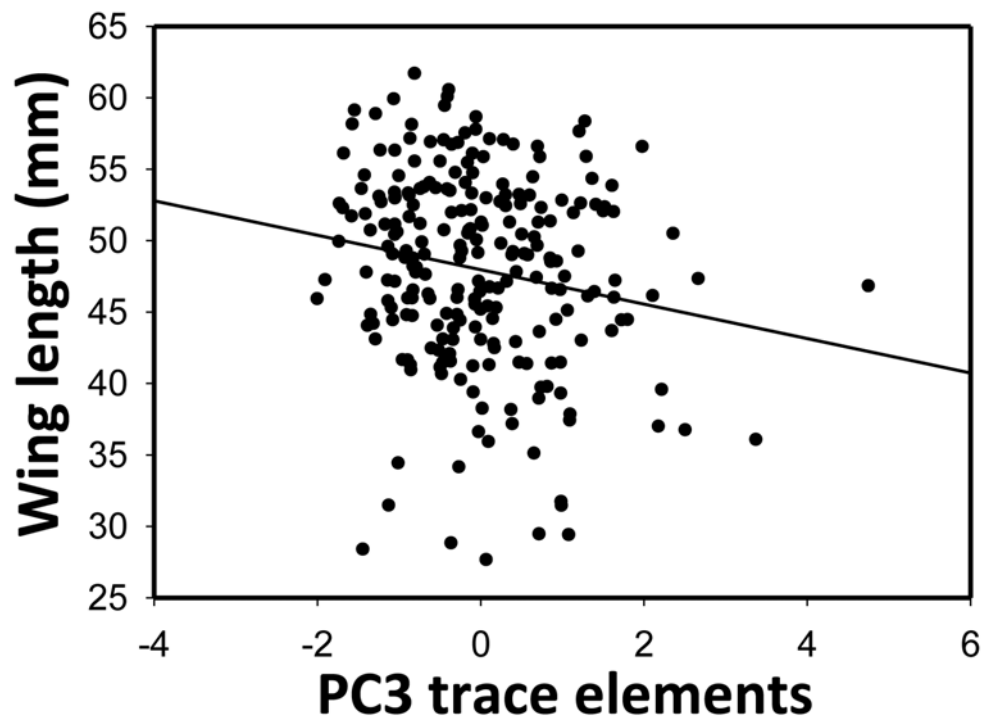
SPATIAL AND TEMPORAL VARIATION IN THE DIET OF TREE SWALLOWS: IMPLICATIONS FOR TRACE ELEMENT EXPOSURE FOLLOWING HABITAT REMEDIATION

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INTRODUCTION

The movement of environmental contaminants through ecosystems and their potential effects on wildlife populations is of great conservation concern. Aquatic ecosystems often become concentrated sources of contaminants due to run-off from terrestrial habitats or industrial disposal practices that use water to dilute or hold contaminated materials (USEPA 2002; Rowe et al. 2002). Thus, much contaminant research focuses on aquatic or piscivorous species and the movement of contaminants through primarily aquatic ecosystems (Krummel et al. 2003; Hasler 1975; Di Giulio and Tillet 1999; Albers et al. 2000). However, freshwater streams produce an incredible biomass of emerging aquatic insects, that can average between 10,000-20,000 individuals/m2/yr (Jackson and Fisher 1986), and lakes typically produce even greater biomass (Gratton and Vander Zanden 2009). As a result, emerging aquatic insects provide important nutrient and energy subsidies to terrestrial insectivores including birds, bats, spiders, and amphibians (Baxter et al. 2005; Sabo and Power 2002; Fukui et al. 2006). Emerging aquatic insects also export contaminants from aquatic systems and could introduce contaminants into terrestrial food webs (Baxter et al. 2005; Vander Zanden and Sanzone 2004).

Terrestrial animals ingesting emerging aquatic insects can be at considerable risk of contaminant exposure and resulting toxicity (Walters et al. 2008; Runck 2007; Menzie 1980; Cristol et al. 2008). Terrestrial predators that forage heavily on aquatic insects in areas contaminated by biomagnifying pollutants such as PCBs and mercury (Hg) (Evers et al. 2005; Kidd et al. 1995) showed significant bioaccumulation of these contaminants that in some cases exceeded the concentrations bioaccumulated by higher trophic level consumers (Cristol et al. 2008). Additional research is needed to address the movement of other contaminants to determine if similar exposure risks exist for lower level trophic consumers as have been found for PCBs and Hg (Chapman et al. 2010).

Pollution of aquatic systems may also alter the quality or quantity of invertebrate prey available to consumers. For example, trace elements can reduce the quantity of invertebrate prey in contaminated areas that can lead to reductions in growth and survival of amphibian and fish larvae (Cherry et al. 1979; Roe et al. 2006; Hopkins et al. 2004). Avian species breeding in areas with metal contamination show dietary shifts to less preferred prey types due to lower abundance and quality of their preferred prey (Eeva et al. 2005). Naturally occurring temporal or spatial variation in emerging aquatic insect hatches or in nutritional requirements during breeding can lead to dietary shifts in animals as well (Morrissey et al. 2010; Dunn and Hannon 1992; Harding 2008; Smits et al. 2005). Dietary shifts could alter the exposure of adults or young to environmental contaminants during key periods of reproduction or development. Thus, it is important to quantify contaminant exposure and the composition of the diet to determine how these factors affect the health of vertebrate consumers.

In December 2008, a coal-fly ash impoundment at the Tennessee Valley Authority (TVA) fossil plant in Kingston, TN ruptured releasing 4.13 million m^3 of coal-fly ash into the Emory River which then flowed into the Clinch and Tennessee Rivers (TVA 2009a). Coal-fly ash contains elevated concentrations of trace elements such as As, Cr, Cu, Cd, Se, and V, but the elements present and concentrations vary depending upon the original composition of the coal and the combustion technologies used (Dvorak 1977, 1978; NRC 2006). Extensive dredging of the Emory River and nearby embayments removed most of the fly-ash from the system by August 2010; however, approximately 400,000 m^3 of ash remains in the system or was left in place to avoid disturbing legacy contamination including Cs-137, PCBs, and Hg in the river sediments (TVA 2009b, 2011b). We examined to what extent trace element contamination from this remediated fly ash spill can move into terrestrial consumers through the consumption of emerging aquatic insects using tree swallows (*Tachycineta bicolor*) as a model system.

Tree swallows are one of the primary model species used to address the movement of contaminants from aquatic to terrestrial ecosystems (Custer 2011). Tree swallows are secondary cavity nesters and readily settle in nest boxes (Robertson et al. 2011). Both sexes remain close to their nest site throughout the breeding season and typically forage within 300-500 m of their box (Dunn and Hannon 1992; Quinney and Ankney 1985). They are aerial insectivores and emerging aquatic insects are thought to be a primary food source when breeding in riparian areas. They lend themselves to dietary studies because adults feeding nestlings retain food in their bill which can be easily removed upon capture (Brasso and Cristol 2008). Insects in a bolus are often still alive and retain diagnostic features that permit identification to order and family.

We collected bolus samples from several tree swallow colonies in the vicinity of the TVA Kingston fly ash spill to identify the types and quantities of insects present in bolus samples. We predicted the tree swallow diet would consist primarily of insects with an aquatic stage in their life cycle because our study sites were located exclusively along shorelines. We also examined the effect of nestling age and seasonality on the composition of tree swallow diet. We predicted that more mayflies would be consumed later in the season and that diet would change little with nestling age, as found in other studies on tree swallows (McCarty and Winkler 1999; Bortolotti et al. 2011). We then tested the hypothesis that composition of the diet affected the exposure of tree swallows to contaminants in the system. We predicted that tree swallows in impacted colonies that consumed more insects with an aquatic stage in their life cycle, particularly Ephemeroptera (mayflies) and Chironomidae (midges), would be exposed to greater concentrations of trace elements than birds that consumed primarily terrestrial insects.

MATERIALS AND METHODS

FIELD SITE AND BOLUS COLLECTION

We established nest box breeding colonies of tree swallows in Roane and Loudoun Counties, TN. These colonies encompassed a trace element contamination gradient that ranged from background exposure at reference colonies to potentially high exposure near the ash spill (Fig. 1, Supp Table 1.). We placed nest boxes at the site of the spill (Spill Site, N = 94) and downstream from this spill at the confluence of the Clinch and Emory Rivers where ash was not dredged during remediation (hereafter Downstream 1, N = 31). Two colonies were monitored further downstream on the Clinch River (Downstream 2) near the Kingston Fossil Plant discharge (N = 31) and Kingston City Park (Downstream 3; N = 43) and were approximately 3 km and 1.5 km from the confluence with the Emory River respectively. Downstream 4 was located about 2.5 km downstream from the confluence of the Clinch and Tennessee Rivers (N = 51). Reference colonies were located at Ft. Loudoun Dam on the Tennessee River approximately 30.5 km east of Kingston (Reference 1, N = 46) and at Tellico Dam (Reference 2, N = 53) on the Little Tennessee River. The third reference colony was located at Long Island (Reference 3, N = 53) about 5.5 km upstream on the Tennessee River from the confluence with the Clinch River. We also placed boxes at Melton Hill Dam (N = 68), 30.5 km northeast of Kingston, TN on the Clinch River. This colony served a role analogous to a positive control because preliminary data gathered prior to this study indicated that tree swallow young and eggs contain elevated concentrations of ash-related contaminants such as Se (ARCADIS 2011). The source(s) of this contamination is unclear, but could include a former coal ash storage pond associated with the Y-12 Security Complex (Cook et al. 1999), the Bull Run Fossil Plant (TVA 2011a; Stantec 2009), or other non-point source pollution (USDA 2009) and warrants further investigation.

Tree swallows were present at the colonies in late February, 2011, and all nest boxes were available to swallows by the end of the first week of March. We placed all boxes within 70 m of the shoreline and 35 m apart

when in a single row or 45 m apart and staggered when in a double row. Tree swallows initiated egg-laying in late April and nestlings were present from mid-May through mid-July 2011. We collected food samples from tree swallows by trapping adults in the nest box immediately after they entered to provision young that were 3-13 days old. Adults continued to hold the food bolus in their bill during trapping, and we used tweezers to remove the sample and place it in a sterile collection bag. Bolus samples were stored in a cooler or refrigerator for up to 8 hours. We transferred each bolus sample into a pre-weighed 1.5 ml Eppendorf tube and obtained the sample wet weight to the nearest 0.1 mg using an analytical balance. Samples were then frozen and stored at -20°C until insect identification.

INSECT IDENTIFICATION

To identify insects, bolus samples were thawed and gently pulled apart using tweezers and rinsing with millipore water in a sterile petri dish. Insects were viewed individually under a compound or dissecting microscope on a sterile glass slide and identified to order. Insects in the orders Diptera, Hymenoptera, Hemiptera, and Coleoptera were identified to family when diagnostic features were available. This was especially critical for Diptera and Coleoptera because only some families possess an aquatic larval stage. We classified families as having an aquatic stage in their life cycle so long as some members of the family exhibited this characteristic and classified them as terrestrial when all members of the family lacked an aquatic stage in their life cycle. It was impossible for us to identify insects further than family due to sample degradation. The number of individuals in each order and family were tabulated as were the number of insects we were unable to identify.

TRACE ELEMENT ANALYSIS

We prepared samples for trace element analysis by placing each bolus in a pre-weighed metal free falcon tube (VWR Scientific, Inc.). The samples were covered with a kimwipe secured with a rubber band and placed in the -80°C freezer for at least 20 minutes before being placed in a freeze drier. Approximately twenty of the samples were reweighed at 24 hour intervals to determine when they were dry based on mass equilibration of the sample. Samples were shipped overnight on dry ice for analysis at the Trace Element Analysis Lab at Dartmouth College.

Samples were digested in the metal-free falcon tubes by open vessel digestion with 0.5 ml 9:1 HNO_3:HCl (Optima, Fisher Scientific, St Louis MO) using microwave heating at 105°C for 45 minutes. After cooling, 0.1 ml H_2O_2 was added to the samples and they were taken through a second heating step. The samples were then diluted to 10 ml with deionized water. The digested samples were analyzed for trace element concentrations by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Concentrations of As, Ba, Cd, Cr, Cu, Fe, Mn, Sr, Tl, V and Zn, (He collision mode), Se (reaction mode), and Hg (normal mode) were quantified in each sample. Digestion quality control measures included digestion blanks, fortified blanks, and reference materials at a frequency of 1 each per twenty samples. There was insufficient bolus material to allow for digestion of duplicates or spikes. Analytical sample duplicates and spikes were performed at a frequency of 1 each per twenty samples. Additional quality control consisted of reporting limit checks, interference checks, and initial and continuing calibration checks and blanks.

Detection limits for each bolus sample varied because the mass of each bolus sample used in the analysis varied. If the trace element concentration was below the detection limit, we assigned that sample a concentration of half of the detection limit for statistical comparisons. The average detection limits (µg/g dry mass) for each element were As 0.014 µg/g, Ba 0.035 µg/g, Cd 0.007 µg/g, Cr 0.415 µg/g, Cu 0.207 µg/g, Fe 6.916 µg/g, Mn 0.069 µg/g, Hg 0.138 µg/g, Se 0.207 µg/g, Sr 0.027 µg/g, Tl 0.007 µg/g, V 0.014 µg/g, and Zn 1.38 µg/g. Trace elements had to be present above the detection limit in more than 50% of the samples at two of the colonies to be included in statistical

models. Only chromium concentrations were below the detection limit in over half of the samples from each colony and were not considered further. Average relative % difference for all 13 trace elements over six analysis duplicates was 12 ± 13%. Average % recovery for 13 trace elements over five analysis spiked samples was 97 ± 21%. Average % recovery for Mn, Fe, Cu, Zn, As, Se, Sr, Cd, and Hg was 100 ± 13% for five separate digestions of NIST 2976, Cr recovery averaged 48%, presumably because the Cr was in a form that is not solubilized by the open vessel acid digestion used here. Other elements analyzed in the bolus samples were not certified in the NIST standard.

ANALYSIS

We tallied the number of each taxon that was collected and the number of times a taxon was present in a bolus sample across the entire study area. We used this to determine what proportion of the tree swallow diet consisted of each taxon and how frequently that taxon was consumed across the entire study area. We also calculated the overall percentage of aquatic insects present in each bolus sample out of the total number of insects that were classified as aquatic or terrestrial in the sample. In addition, we also examined the effects of nestling age and season on the composition of bolus samples.

To examine diet composition among colonies, we limited our analysis to locations where we collected a minimum of four bolus samples. We obtained only three bolus samples from Reference 3 and so excluded that colony from the analysis. For each bolus sample, we calculated the proportion of insects with an aquatic life-stage and the proportion of the sample that consisted of the groups: Diptera other than Chironomidae, Diptera family Chironomidae, Hymenoptera, Hemiptera, Coleoptera, and proportion other taxa out of the total number of insects present in each sample. We selected these taxa because they appeared to be an important part of the tree swallow diet based on their occurrence in bolus samples (Table 1). Because these proportions are not independent of each other and must sum to one, we used compositional analysis to produce five new variables that were linearly independent of each other (Aitchison 1986). We did this by log-transforming the quotient produced by dividing the proportion of a sample that consisted of a taxon of interest by the proportion of that sample classified as other. In cases where the proportion was 0 (and therefore could not be log-transformed), we added 0.005, to the sample proportion which was an order of magnitude smaller than our smallest proportion (0.015). The log-transformed variables were used to compare diet composition among colonies (see below).

We log transformed all elemental concentrations because the data were not normally distributed. Concentrations of some trace elements were highly correlated so we used principal components analysis (PCA) to produce three principal components of trace element concentrations. We performed multivariate ANOVAs on the principal components to compare bolus composition or trace element concentrations among colonies. We included nestling age and Julian sample collection date as covariates in the model, but removed them from the final model if $p > 0.10$ and checked that these covariates did not violate the assumption of slope homogeneity. We expected trace element exposure would vary with the composition of the diet, so we performed three backward linear regressions with the transformed prey composition proportions as independent variables and the principal components of log-transformed trace element concentrations as the dependent variables. Variables were removed from the model if $p > 0.10$. All statistical tests are two-tailed and we set $\alpha = 0.05$. All statistical analyses were performed using PASW 18 (SPSS 2009).

RESULTS

COMPOSITION OF BOLUS SAMPLES AND TEMPORAL CHANGES IN DIET

We collected 109 bolus samples across the study site and samples ranged in size from a single insect to 170 insects (average number of insects per bolus = 33.7 ± 3.4, median= 23). We were able to classify to order 3675 arthropods and were unable to identify 38 due to degradation. The tree swallow diet consisted primarily of Dipterans; this order comprised 77.6% (n = 2853) of the total number of insects collected and were present in 99 of the 109 (90.8 %) of the bolus samples (Table 1). The family Chironomidae, which includes members with an aquatic larval stage, made up 59.5% (n = 2187) of the total arthropods collected in bolus samples and was present in 65.1% of the bolus samples collected (n = 71). Several other Dipteran families that have an aquatic stage in their life cycle appeared in over 10% of the bolus samples collected, and included the families Culicidae, Dolichopodidae, Empididae, Phoridae, Simuliidae, and Syrphidae. The orders Hymenoptera, Hemiptera, Coleoptera, and Trichoptera were present in over 12% of bolus samples but only Trichopterans (caddisflies) and some Coleopteran families have an aquatic life cycle stage. However, the Coleopteran families with an aquatic larval stage represented a small proportion of insects consumed by the swallows (Table 1). Of the 3675 insects classified, 2473 (67.3%) had an aquatic stage in their life cycle, representing a large proportion of the tree swallow diet. We focused subsequent analyses on proportions of non-Chironomidae Diptera, Chironomidae, Hemiptera, Hymenoptera, and Coleoptera because each of these taxa was present in over 20% of the bolus samples and represented a large part of the tree swallow diet.

We also examined seasonal and age-related changes in diet by examining correlations between these variables and the proportion of the diet that consisted of the five taxa predominantly consumed by tree swallows. Bolus samples collected later in the season had a greater proportion of Hymenoptera (r = 0.294, n = 108, p = 0.002), but the proportions of non-Chironomidae Diptera (r = 0.035, n = 108, p = 0.91), Chironomidae (r = -0.039, n = 108, p = 0.69), Hemiptera (r = -0.068, n = 108, p = 0.49) and Coleoptera (r = 0.011, n = 108, p = 0.91) did not change with season. We also examined the consumption of Ephemeroptera and it did not change seasonally (r = 0.116, n = 108, p = 0.23). We found that bolus composition changed with nestling age. The proportion of the diet that consisted of non-Chironomidae Diptera (r = -0.324, n = 106, p = 0.001), Hymenoptera (r = -0.193, n = 106, p = 0.05), and Coleoptera (r = -0.202, n = 106, p = 0.04) decreased with nestling age, but the proportion of the diet consisting of Chironomidae (r = -0.007, n = 106, p = 0.94) and Hemiptera (r = -0.057, n = 106, p = 0.56) remained nearly constant with nestling age.

COLONY EFFECTS ON BOLUS COMPOSITION

We examined the influence of location on the composition of bolus samples collected from tree swallows and included nestling age as a covariate in these models. We found that location significantly influenced the proportion of the diet that consisted of Chironomidae ($F_{7, 97}$ = 3.556, p = 0.002, Fig. 2) and Hemiptera ($F_{7, 97}$ = 2.808, p = 0.01) with nearly significant differences for non-Chironomidae Dipterans ($F_{7, 97}$ = 1.926, p = 0.07) and Coleopterans ($F_{7, 97}$ = 1.840, p = 0.09). Nestling age had a significant effect on the consumption of non-Chironomidae Diptera ($F_{1, 93}$ = 8.044, p = 0.006) and sample collection date had a significant effect on the consumption of Hymenoptera ($F_{1, 93}$ = 9.846, p = 0.002). Post-hoc tests revealed that colony-related differences were due to the increased consumption of Chironomidae at the Spill Site compared to Downstream 3 (p = 0.014) and Downstream 4 (p = 0.031), as well as some evidence of greater consumption of Chironomidae at Melton Hill Dam than at Downstream 3 (p= 0.078).

Significantly fewer Hemiptera were consumed at Downstream 3 than at Reference 1 ($p = 0.027$) or at Downstream 1 ($p = 0.039$). We found no significant differences among colonies in the proportion of Hymenoptera consumed ($F_{7, 97} = 0.802$, $p = 0.587$). We also found that the proportion of insects with an aquatic stage in their life cycle consumed by tree swallows differed significantly among colonies ($F_{7, 97} = 3.921$, $p = 0.001$, Fig. 3). A greater proportion of insects with an aquatic stage in their life cycle were consumed at Melton Hill Dam compared to Downstream 3 ($p = 0.005$), Downstream 4 ($p = 0.010$) and Reference 1 ($p = 0.087$). More insects with an aquatic larval stage were consumed at the Spill Site compared to Downstream 3 ($p = 0.036$) and Downstream 4 ($p = 0.054$).

COLONY AND DIET EFFECTS ON TRACE ELEMENT CONCENTRATIONS

The principal components analysis produced three principal components (PC) that together explained 60.5% of the variance in trace element concentrations (Table 2, Supp Table 2). PC1 received high, positive factor loadings for Ba, Cu, Cd, Fe, Mn, Sr, Se, and Zn and explained 33.1% of the variance in trace element concentrations. PC2 received high, positive factor loadings for As and V, while PC3 had high, positive loadings for Tl and Hg. PC2 explained an additional 17.1% of the variance and PC3 an additional 10.3% of the variance in trace element concentrations.

We found significant differences among colonies in PC1 ($F_{7, 96} = 3.359$, $p = 0.003$) and PC3 ($F_{7, 96} = 2.977$, $p = 0.007$), while PC2 ($F_{7, 96} = 1.741$, p = 0.109) did not differ among colonies (Fig. 4). These results indicated that greater concentrations of contaminants were present in bolus samples at the Spill Site than at the other colonies. For PC1, post-hoc tests revealed that the Spill Site had significantly higher PC1 scores than Reference 1 ($p = 0.019$), Downstream 2 ($p = 0.028$), and Downstream 4 ($p = 0.020$), and suggested a trend for PC1 scores to be higher than at Downstream 3 ($p = 0.062$). PC3 scores were significantly lower at Melton Hill than at the Spill Site ($p = 0.030$) and Downstream 4 ($p = 0.003$). However, concentrations of most trace elements were below levels of toxicological concern (Table 3).

The proportion of the diet that consisted of aquatic insects was positively related to the exposure of tree swallows to contaminants associated with PC1 ($r^2 = 0.191$, $F_{1, 104} = 24.61$, $p < 0.001$, Fig. 5a). The proportion of the diet that consisted of aquatic insects was unrelated to exposure to contaminants associated with PC2 ($r^2 = 0.007$, $F_{1, 104} = 0.705$, $p = 0.403$) and with PC3 ($r^2 = 0.0001$, $F_{1, 104} = 0.015$, $p = 0.903$). When we focused on diet composition, exposure to contaminants associated with PC1 was best explained by a model that included the proportion of Chironomidae and Hemiptera in the diet (Fig. 5b, c, $r^2 = 0.372$, $F_{2, 103} = 30.55$, $p < 0.001$). Examining each taxon separately, we found that consumption of Chironomidae was positively related to contaminant exposure ($r^2 = 0.338$, $F_{1, 104} = 53.21$, $p < 0.001$) while consumption of Hemiptera was weakly negatively related to exposure ($r^2 = -0.041$, $F_{1, 104} = 4.415$, $p = 0.038$). For PC2, we found a weak but nearly significant negative relationship between the proportion of Hymenoptera in the bolus sample and PC2 levels ($r^2 = -0.035$, $F_{1, 104} = 3.806$, $p = 0.054$). We found no relationship between diet and PC3 ($r^2 = 0.003$, $F_{1, 104} = 0.313$, $p = 0.577$).

DISCUSSION

We examined the diet of tree swallows to determine whether trace elements from a recently remediated coal-fly ash spill were entering the terrestrial ecosystem through the consumption of emerging aquatic insects. Across the entire study site, over half of the tree swallow diet consisted of insects that had an aquatic stage in their life cycle. Dipterans, particularly members of the family Chironomidae, which have an aquatic stage in their life cycle, were

consumed in the largest quantities by tree swallows. Hymenopterans, Hemipterans, and Coleopterans were also brought to young in appreciable quantities. Thus, looking at our results across the entire study site, it appears that tree swallows have the capacity to be exposed to trace elements through the consumption of insects with an aquatic larval stage, particularly due to the consumption of Chironomidae. However, we found significant differences among colonies in the proportion of the diet that consisted of insects with an aquatic stage in their life cycle. Tree swallows at the Spill Site and Melton Hill Dam consumed more insects with an aquatic larval stage causing birds at these colonies to potentially be exposed to more aquatic contaminants than at other colonies. This result was caused largely by greater consumption of Chironomidae at these two colonies compared to others.

A greater proportion of the diet consisted of Hemiptera at Downstream 1 and at Reference 1 compared to the other colonies. This dietary difference is likely due to naturally occurring habitat differences because Reference 1 and Downstream 1 are located near large fields with deep grass while Melton Hill Dam and the Spill Site are essentially well-maintained lawns that may provide poor habitat for terrestrial insects. Reference 1 may provide poor aquatic habitat for insects as well, as this site is adjacent to a marina. The lack of consumption of Chironomidae at Downstream 1 is odd given that surveys of nearby sections of the Emory River detected numerous Chironomidae larvae (Baker 2011). It is unclear what factors may have limited the consumption of Chironomidae at this colony or if it is the result of sampling error caused by the small number of bolus samples collected at this colony. Aquatic substrate, habitat heterogeneity, water depth, water temperature, and pollutants all can affect aquatic insect diversity and could explain the differences detected between Melton Hill Dam, Reference 1, Downstream 1, and the Spill Site (Rosa et al. 2011; Kasangaki et al. 2006; Wright and Burgin 2009; Odume and Muller 2011). In the future, it would be worthwhile to better quantify habitat differences and how they affect the insect taxa present at a colony.

We found few seasonal changes in the tree swallow diet. The proportion of the diet consisting of Hymenoptera increased later in the season, but the consumption of other taxa did not change appreciably with season. While Ephemeroptera were not consumed in large quantities in this study, other studies have indicated that they can be an important component of the tree swallow diet and that their consumption increases seasonally (Smits et al. 2005; Papp et al. 2007). However, we collected few bolus samples that contained mayflies despite the incredible quantity available. One reason for this is that many tree swallows had fledged their first brood before the majority of mayflies emerged. Studies in other systems have detected annual variation in the consumption of mayflies that was related to variation in weather conditions (Smits et al. 2005). It is possible in this system that greater numbers of Ephemeroptera would be consumed in a year when mayfly hatches better coincide with tree swallow nesting phenology.

We also found that nestling age significantly affected the proportion of the diet that consisted of non-Chironomidae Diptera, Hymenoptera, and Coleoptera. In all cases, the proportion of these taxa in the diet decreased with nestling age. However, other studies on tree swallows have detected little change in diet with nestling age (Bortolotti et al. 2011; McCarty and Winkler 1999). It is possible that the changes we detected were the result of short-term variation in the availability of prey that was not detected by our seasonal analysis. It is unlikely that this variation in the diet would lead to age-specific exposure to contaminants because the proportion of the diet consisting of Chironomidae remained fairly constant with nestling age. This taxon comprised a major portion of tree swallow diet, and could be the primary route of trace element exposure to tree swallows in this system.

Bolus samples from the Spill Site had significantly higher PC1 scores than many of the reference and downstream colonies. This indicates that birds at the Spill Site are exposed to greater concentrations of trace elements including Se, Sr, and Fe than birds at other colonies. The Spill Site also had significantly higher PC3 scores than Melton Hill Dam but so did a low impacted colony, Downstream 4. Elevated PC3 scores from Downstream 4 could be related to residual contamination from the spill, but are more likely the result of atmospheric deposition of

contaminants (Hg) or the result of other sources of contamination upstream on the Tennessee River. Reference 3 is located a few km upstream from Downstream 4 on the Tennessee River, but we were unable to examine bolus contaminant concentrations at Reference 3 due to a small sample size. Thus, we were unable to determine if trace elements were present in the tree swallow diet in nearby portions of the Tennessee River or were only found in the vicinity of the spill.

One of the most important findings of our study revealed the importance of aquatic insects as vectors of contamination to terrestrial consumers. Tree swallows whose diet consisted predominantly of insects with an aquatic stage in their life cycle were exposed to higher levels of contaminants associated with PC1. In particular, the consumption of Chironomidae led to greater exposure to trace elements associated with the fly ash spill while consumption of Hemiptera and Hymenoptera were negatively related to trace element exposure. Although tree swallows consuming insects with an aquatic larval stage were exposed to higher concentrations of contaminants, the trace element concentrations were low and below levels typically associated with adverse reproductive effects. For instance, Hg concentrations in tree swallow bolus samples from a highly contaminated site in Virginia (Brasso and Cristol 2008) were over ten times higher than the Hg concentrations detected at the Spill Site. The highest dietary concentrations of Se in our study occurred at the Spill Site and were at the lower limit of the dietary concentration range thought to result in reproductive effects in avian species (Ohlendorf and Heinz 2011). Most of the trace element concentrations detected in tree swallow bolus samples were an order of magnitude lower than concentrations detected in a study conducted on common grackles (*Quiscalus quiscala*) breeding near an active ash settling basin (Bryan et al. 2012). Indeed, concentrations of many of the trace elements in our study were similar to another study on tree swallows which found no effects of trace element contamination on reproductive success or oxidative stress (Custer et al. 2006).

Our study demonstrates that emerging aquatic insects can transport trace elements to terrestrial consumers such as tree swallows. This suggests that other terrestrial insectivores could be at risk of exposure to trace elements from coal-fly ash. However, we detected low levels of contaminants in the insects that we collected from this particular system. This is likely the result of successful remediation efforts that occurred in the three years preceding our study, and/or to the effects of a lotic, rather than lentic, environment. We also found that the tree swallow diet varied substantially in habitats that appeared superficially to be quite similar. Despite locating boxes strategically within 70 m of the shore, tree swallows in some colonies foraged extensively on terrestrial insects, possibly limiting their exposure to aquatic contaminants. Our results suggest that is some systems it may be problematic to assume uniform consumption of particular types of prey or to infer exposure to aquatic contaminants solely by monitoring birds in riparian areas. We suggest that future studies examining the movement of aquatic contaminants into terrestrial consumers include dietary analysis to confidently document routes of exposure.

REFERENCES

Aitchison J (1986) The statistical analysis of compositional data. Monographs on statistics and applied probability. Chapman and Hall, New York.

Albers PH, Heinz GH, Ohlendorf HM (2000) Environmental contaminant and terrestrial vertebrates: Effects on populations, communities, and ecosystems. SETAC Press, Pensacola, FL, USA.

ARCADIS (2011) Draft Trace Element Concentrations in Tree Swallows: 2009-2010. vol November 2011. TVA, Kingston.

Baker TF (2011) Evaluation of the benthic macroinvertebrate community in the vicinity of TVA's Kingston Fossil Plant, 2009-2010. TVA, Chattanooga, TN.

Baxter CV, Fausch KD, Saunders WC (2005) Tangled webs: reciprocal flows of invertebrate prey link streams and riparian zones. Freshwater Biol 50:201-220.

Bortolotti LE, Harriman VB, Clark RG, Dawson RD (2011) Can changes in provisioning by parent birds account for seasonally declining patterns of offspring recruitment? Can J Zool 89:921-928.

Brasso RL, Cristol DA (2008) Effects of mercury exposure on the reproductive success of tree swallows (*Tachycineta bicolor*). Ecotoxicology 17:133-141.

Bryan AL, Hopkins WA, Parkikh JH, Jackson BP, Unrine JM (2012) Coal fly ash basins as an attractive nuisance to birds: Parental provisioning exposes nestlings to harmful trace elements. Environ Pollut 161:170-177.

Chapman PM, Adams WJ, Brooks ML, Delos CG, Luoma SN, Maher WA, Ohlendorf HM, Presser TS, Shaw DP (eds) (2010) Ecological Assessment of Selenium in the Aquatic Environment. CRC Press, New York, New York.

Cherry DS, Guthrie RK, Sherberger FF, Larrick SR (1979) Influence of coal ash and thermal discharges upon the distribution and bioaccumulation of aquatic invertebrates. Hydrobiologia 62:257-267.

Cook RB, Suter GW, Sain ER (1999) Ecological risk assessment in a large river-reservoir: 1. Introduction and background. Environ Toxicol Chem 18:581-588.

Cristol DA, Brasso RL, Condon AM, Fovargue RE, Friedman NR, Hallinger KK, Monroe AP, White AE (2008) The movement of aquatic mercury through terrestrial food webs. Science 320:335.

Custer CM (2011) Swallows as sentinel species for contaminant exposure and effect studies. In: JE E, CA M, CA B (eds) Wildlife Ecotoxicology: Forensic Approaches. Springer, New York, pp 45-91.

Custer CM, Custer TW, Warburton D, Hoffman DJ, Bickham JW, Matson CW (2006) Trace element concentrations and bioindicator response in tree swallows from northwestern Minnesota. Environ Monit Assess 118:247-266.

Di Giulio RT, Tillet DE (1999) Reproductive and developmental effects of contaminants in oviparous vertebrates. SETAC Press, Pensacola, FL, USA.

Dunn PO, Hannon SJ (1992) Effects of food abundance and male parental care on reproductive success and monogamy in tree swallows. Auk 109:488-499.

Dvorak AJ (1977) The enviromental effects of using coal for generating electricity. vol NUREG-0252. U. S. Nuclear Regulatory Commission, Washington, DC.

Dvorak AJ (1978) Impacts of coal-fired power plants on fish, wildlife, and their habitats. vol FWS/OBS-78/29. U. S. Fish and Wildlife Service, Behesda, Maryland.

Eeva T, Ryömä M, Riihimäki J (2005) Pollution-related changes in the diets of two insectivorous passerines. Oecologia 145:629-639.

Fukui D, Murakami M, Nakano S, Aoi T (2006) Effects of aquatic insects on bat foraging in a riparian forest. J Anim Ecol 75:1252-1258.

Gratton C, Vander Zanden MJ (2009) Flux of aquatic insect productivity to land: comparison of lentic and lotic systems. Ecology 90:2689-2699.

Harding LE (2008) Non-linear uptake and hormesis effects of selenium in red-winged blackbirds (*Agelaius phoeniceus*). Sci Total Environ 389:350-366.

Hasler AD (1975) Coupling of land and water systems. Springer-Verlag, New York, New York USA.

Hopkins WA, Staub BP, Snodgrass JW, Taylor BE, DeBiase AE, Roe JH, Jackson BP, Congdon JD (2004) Responses of benthic fish exposed to contaminants in outdoor microcosms - examining the ecological relevance of previous laboratory toxicity tests. Aquat Toxicol 68:1-12.

Jackson JK, Fisher SG (1986) Secondary production, emergence, and export of aquatic insects of a Sonoran Desert stream. Ecology 67:629-638.

Kasangaki A, Babaasa D, Efitre J, McNeilage A, Bitariho R (2006) Links between anthropogenic perturbations and benthic macroinvertebrate assemblages in afromontane forest streams in Uganda. Hydrobiologia 563:231-245.

Krummel EM, MacDonald RW, Kimpe LE, Gregory-Eaves I, Demers MJ, Smol JP, Finney B, Blais JM (2003) Delivery of pollutants by spawning salmon: fish dump toxic industrial compounds in Alaskan lakes on their return from the ocean. Nature 425:255-256.

McCarty JP, Winkler DW (1999) Foraging ecology and diet selectivity of tree swallows feeding nestlings. Condor 101:246-254.

Menzie CA (1980) Potential significance of insects in the removal of conaminants from aquatic systems. Water Air Soil Poll 13:473-479.

Morrissey CA, Elliott JE, Ormerod SJ (2010) Diet shifts during egg laying: Implications for measuring contaminants in bird eggs. Environ Pollut 158:447-454.

NRC (2006) Managing coal combustion residues in mines. National Academies Press, Washington, D.C.

Odume ON, Muller WJ (2011) Diversity and structure of Chironomidae communities in relation to water quality differences in the Swartkops River. Phys Chem Earth 36:929-938.

Ohlendorf HM, Heinz GH (2011) Selenium in birds. In: Beyer WN, Meador JP (eds) Environmental Contaminants in Biota. CRC Press, Boca Raton, pp 669-701.

Papp Z, Bortolotti G, Sebastian M, Smits J (2007) PCB Congener Profiles in Nestling Tree Swallows and Their Insect Prey. Arch Environ Contam Toxicol 52:257-263.

Quinney TE, Ankney CD (1985) Prey size selection by tree swallows. Auk 102:245-250.

Robertson RJ, Stutchbury BJ, Cohen RR, Winkler DW, Hallinger KK, Ardia DR (2011) Tree Swallow. Cornell Lab of Ornithology. http://bna.birds.cornell.edu/bna/species/011/articles/introduction. Accessed April 10 2012

Roe JH, Hopkins WA, DuRant SE, Unrine JA (2006) Effects of competition and coal-combustion wastes on recruitment and life history characteristics of salamanders in temporary wetlands. Aquat Toxicol 79:176-184.

Rosa B, de Oliveira VC, Alves RD (2011) Structure and spatial distribution of the Chironomidae community in mesohabitats in a first order stream at the Poco D'Anta Municipal Biological Reserve in Brazil. J Insect Sci 11.

Rowe CL, Hopkins WA, Congdon JD (2002) Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: A review. Environ Monit Assess 80:207-276.

Runck C (2007) Macroinvertebrate production and food web energetics in an industrially contaminated stream. Ecol Appl 17:740-753.

Sabo JL, Power ME (2002) River-watershed exchange: Effects of riverine subsidies on riparian lizards and their terrestrial prey. Ecology 83:1860-1869.

Smits JEG, Bortolotti GR, Sebastian M, Ciborowski JJH (2005) Spatial, temporal, and dietary determinants of organic contaminants in nestling tree swallows in Point Pelee National Park, Ontario, Canada. Environ Toxicol Chem 24:3159-3165.

SPSS (2009) PASW Statistics GradPack 18. SPSS Inc, Chicago.

Stantec (2009) TVA disposal facility assessment phase 1 plant summary Bull Run Fossil Plant (BRF). Stantec, Nashville, TN.

TVA (2009a) Corrective Action Plan for the TVA Kingston Fossil Plant Ash Release. http://www.tva.gov/kingston/admin_record/pdf/G/G4.pdf.

TVA (2009b) Emergency Dredging for the Kingston Fossil Plant Ash Dike Failure Roane County, TN E. TVA, Kingston.

TVA (2011a) Inspection report: TVA's groundwater monitoring at coal combustion products disposal areas. vol Inspection 2009-12991. Office of Inspector General, Tennessee Valley Authority, Knoxville, TN.

TVA (2011b) TVA Kingston Fossil Fuel Plant Release Site On-Scene Coordinator Report for the Time-Critical Removal Action May 11, 2009 through December 2010. TVA, Harriman, TN.

USDA (2009) Lower Clinch River rapid watershed assessment. USDA and NRCS, TN.

USEPA (2002) National water quality inventory report. vol EPA-841-R-02-001. Washington, D.C. .

Vander Zanden MJ, Sanzone DM (2004) Food web subsidies at the land-water ecotone. In: Polis GA, Power ME, Huxel GR (eds) Food Webs at the Landscape Level. University of Chicago Press, Chicago, Illinois, USA, pp 185-188.

Walters DM, Fritz KM, Otter RR (2008) The dark side of subsidies: Adult stream insects export organic contaminants to riparian predators. Ecol Appl 18:1835-1841.

Wright IA, Burgin S (2009) Effects of organic and heavy metal pollution on chironomids within a pristine upland catchment. Hydrobiologia 635:15-25.

TABLES AND FIGURES

TABLE 1.

We collected a total of 109 bolus samples from tree swallows as they provisioned their young. The common name of the taxa is given in parentheses next to the scientific name. Taxa with an aquatic stage in their life cycle are denoted by an A and those with a strictly terrestrial life cycle by a T. Taxa in bold made up greater than 1% of the total arthropods sampled and/or were present in more than 10% of the bolus samples collected. Unknown denotes individuals identified to order but that could not be accurately identified to family.

Order Family	# of arthropods	% Arthropods	# bolus samples taxon present in	% of bolus samples taxon present in
Diptera (flies)	**2853**	**77.63%**	**99**	**90.82%**
Unknown Diptera	284		59	
Agromyzidae (lead miner flies) (T)	5		4	
Anisopodidae (wood gnats) (T)	5		4	
Bibionidae (March flies)(T)	**44**	**1.20%**	**3**	**2.75%**
Calliphoridae (blow flies)(T)	2		2	
Ceratopogonidae (biting midges)(A)	3		2	
Cecidomyiidae (gall midges)(T)	4		3	
Chironomidae (midges)(A)	**2187**	**59.51%**	**71**	**65.14%**
Chloropidae (fruit flies)(T)	14		9	
Culicidae (mosquitoes)(A)	**21**	**<1%**	**13**	**11.93%**
Dolichopodidae (longlegged flies) (A)	**17**	**<1%**	**12**	**11.01%**
Drosophilidae (vinegar flies)(T)	13		5	
Empididae (dance flies)(A)	**27**	**<1%**	**18**	**16.51%**
Ephydridae (shore flies)(A)	3		2	
Milichiidae (freeloader flies)(T)	6		3	
Muscidae (house flies)(A)	5		5	
Mycetophilidae (fungus gnats)(T)	**15**	**<1%**	**14**	**12.85%**
Phoridae (scuttle flies)(A)	**14**	**<1%**	**11**	**10.09%**
Platystomatidae (signal flies)(T)	1		1	
Sarcophagidae (flesh flies)(A)	6		6	
Scatopsidae (dung midges)(T)	4		3	
Scathophagidae (dung flies)(A)	3		1	
Sciaridae (dark-winged fungus gnats)(T)	4		4	
Sepsidae (black scavenger flies)(T)	9		7	
Simuliidae (black flies)(A)	**81**	**2.20%**	**20**	**18.35%**
Sphaeroceridae (lesser dung flies)(T)	7		4	
Stratiomyidae (soldier flies)(A)	22		10	
Syrphidae (flower flies)(A)	**31**	**<1%**	**16**	**14.68%**

Tachinidae (tachinid flies)(T)	9		9	
Tephritidae (fruit flies)(T)	2		2	
Tipulidae (crane flies)(A)	2		2	
Xylomyidae (wood soldier flies)(T)	1		1	
Xylophagidae (awl flies)(T)	2		1	
Hymenoptera (wasps, bees, ants)	**237**	**6.45%**	**46**	**42.20%**
Unknown Hymenoptera	26		19	
Braconidae (Braconid parasitoid wasps)(T)	19		9	
Chalcidoidea (Chalcid parasitoid wasps)(T)	4		4	
Diapriidae (Diaprid parasitoid wasps)(T)	1		1	
Formicidae (ants)(T)	**141**	**3.84%**	**22**	**20.18%**
Ichneumonidae (Ichneumon parasitoid wasps)(T)	11		8	
Scelionidae (parasitoid wasps)(T)	34		4	
Tenthredinidae (common sawflies)(T)	1		1	
Hemiptera (true bugs& leaf hoppers)	**388**	**10.56%**	**66**	**60.55%**
Unknown Hemiptera	4		4	
Aphididae (aphids)(T)	**239**	**6.50%**	**44**	**40.37%**
Cercopidae (froghoppers)(T)	**76**	**2.07%**	**18**	**16.51%**
Cicadellidae (leafhopper)(T)	**42**	**1.14%**	**25**	**22.94%**
Delphacidae (planthoppers)(T)	1		1	
Flatidae (flatid planthoppers)(T)	1		1	
Membracidae (treehopper)(T)	1		1	
Miridae (leaf bugs)(T)	9		7	
Psyllidae (jumping plant lice)(T)	13		9	
Saldidae (shore bugs)(T)	1		1	
Tingidae (lace bugs)(T)	1		1	
Ephemeroptera (mayflies)(A)	13	<1%	7	6.42%
Isoptera (termites)(T)	**41**	**1.12%**	**5**	**4.59%**
Plecoptera (stoneflies)(A)	1	<1%	1	<1%
Psocoptera (barkflies)(T)	5	<1%	4	3.67%
Lepidoptera (moths & butterflies)(T)	8	<1%	6	5.51%
Coleoptera (beetles)	**57**	**1.55%**	**26**	**23.85%**
Unknown Coleoptera	12		9	
Carabidae (ground beetle)(T)	2		2	
Chrysomelidae (leaf beetle)(T)	1		1	
Ciidae (minute tree fungus beetle)(T)	1		1	
Curculionidae (snout beetles)(T)	16		8	
Elateridae (click beetles)(T)	1		1	
Histeridae (clown beetles)(A)	7		3	
Hydrophilidae (water scavenger beetles)(A)	1		1	

Mordellidae (tumbling flower beetles)(T)	1		1	
Rhipiphoridae (wedge-shaped beetles)(T)	1		1	
Scarabaeidae (scarab beetles)(T)	3		2	
Scolytidae (bark beetle)(T)	6		2	
Staphylinidae (rove beetles)(T)	4		4	
Tenebrionidae (darkling beetles)(T)	1		1	
Tricoptera (caddisflies)(A)	**29**	**<1%**	**15**	**13.76%**
Arachinda (spiders)(T)	5	<1%	4	3.67%
Total arthropods	3675			

TABLE 2.

Factor loadings for the PCA analysis of trace element concentrations. Trace element concentrations were log transformed to improve normality prior to performing the principal components analysis.

Trace Element	PC1	PC2	PC3
Arsenic	0.553	0.623	-0.072
Iron	0.822	-0.151	0.245
Barium	0.552	0.143	-0.369
Cadmium	0.677	-0.166	0.166
Copper	0.556	-0.515	-0.312
Manganese	0.579	-0.452	-0.018
Mercury	0.114	0.311	0.697
Selenium	0.699	0.139	0.166
Strontium	0.763	0.367	-0.120
Thallium	0.026	0.085	0.551
Vanadium	0.368	0.750	-0.233
Zinc	0.603	-0.548	0.135
Eigen value	3.972	2.050	1.231
% variance	33.10	17.08	10.25

TABLE 3.

Univariate mean and standard error of trace element concentrations in tree swallow bolus samples by location (µg/mg).

E	Reference 1	Reference 2	Melton Hill Dam	Spill Site	Downstream 1	Downstream 2	Downstream 3	Downstream 4
As	0.07 ± 0.03	0.10 ± 0.03	0.17 ± 0.07	0.24 ± 0.07	0.04 ±0.02	0.10 ± 0.03	0.13 ± 0.02	0.10 ± 0.04
Ba	2.1 ± 0.3	4.7 ± 1.4	13.3 ± 3.8	8.0 ± 3.2	15.8 ± 9.6	3.19 ± 1.2	12.25 ± 10.5	4.46 ± 2.2
Cd	0.27 ± 0.05	0.40 ± 0.11	0.40 ± 0.05	0.50 ± 0.09	0.66 ± 0.49	0.24 ± 0.09	0.57 ± 0.25	0.29 ± 0.12
Cu	19.7 ± 2.4	24.2 ± 10.0	19.9 ± 2.9	19.6 ± 2.1	28.1 ± 13.1	15.9 ± 7.5	14.9 ± 1.7	12.7 ± 2.6
Fe	186.0 ± 50.9	192.5 ± 64.3	130.5 ± 18.8	380.4 ± 49.6	138.6 ± 41.7	85.8 ± 23.0	139.8 ± 38.6	120.5 ± 26.5
Mn	73.0 ± 37.8	48.2 ± 22.0	44.2 ± 8.2	37.4 ± 4.7	42.7 ± 13.5	15.0 ± 5.3	29.6 ± 7.5	47.5 ± 20.1
Hg	0.08 ± 0.01	0.09 ± 0.04	0.06 ± 0.01	0.11 ± 0.01	0.09 ± 0.02	0.11 ± 0.03	0.06 ± 0.01	0.11 ± 0.03
Se	0.63 ± 0.23	0.65 ± 0.27	2.07 ± 0.54	3.07 ± 0.39	0.67 ± 0.32	1.51 ± 0.74	1.23 ± 0.29	0.67 ± 0.18
Sr	1.7 ± 0.5	3.2 ± 0.8	4.8 ± 1.0	17.0 ± 11.4	3.3 ± 1.3	3.4 ± 1.5	3.1 ± 1.3	2.5 ± 1.0
Tl	0.01 ± 0.006	0.01 ± 0.002	0.01 ± 0.002	0.02 ± 0.003	0.01 ± 0.001	0.02 ± 0.011	0.01 ± 0.008	0.10 ± 0.070
V	0.01 ± 0.003	0.02 ± 0.003	0.05 ± 0.026	0.20 ± 0.151	0.01 ± 0.002	0.05 ± 0.031	0.05 ± 0.027	0.02 ± 0.012
Zn	122.8 ± 14.7	127.5 ± 24.9	111.1 ± 10.5	113.6 ± 9.74	257.6 ± 118.7	70.8 ± 17.4	112.7 ± 14.7	114.8 ± 22.2

FIGURE 1.

Tree swallow colonies located near Kingston, TN. The study area consisted of one highly impacted colony located at the site of the spill (Spill Site, SS) and a second highly impacted colony (Downstream 1 or D1) located at the confluence of the Emory and Clinch Rivers. Moderately impacted colonies were located on the Clinch River at D2 and D3 and a low impacted colony was located downstream on the Tennessee River (D4). Two reference colonies were located near Lenoir City, TN at Ft. Loudoun Dam (R1) and Tellico Dam (R2) as well as a positive control located at Melton Hill Dam is not pictured here. A third reference colony was located on Long Island (R3) on the Tennessee River upstream from the confluence with the Clinch River. River kilometers are given in each river to indicate distances among colonies.
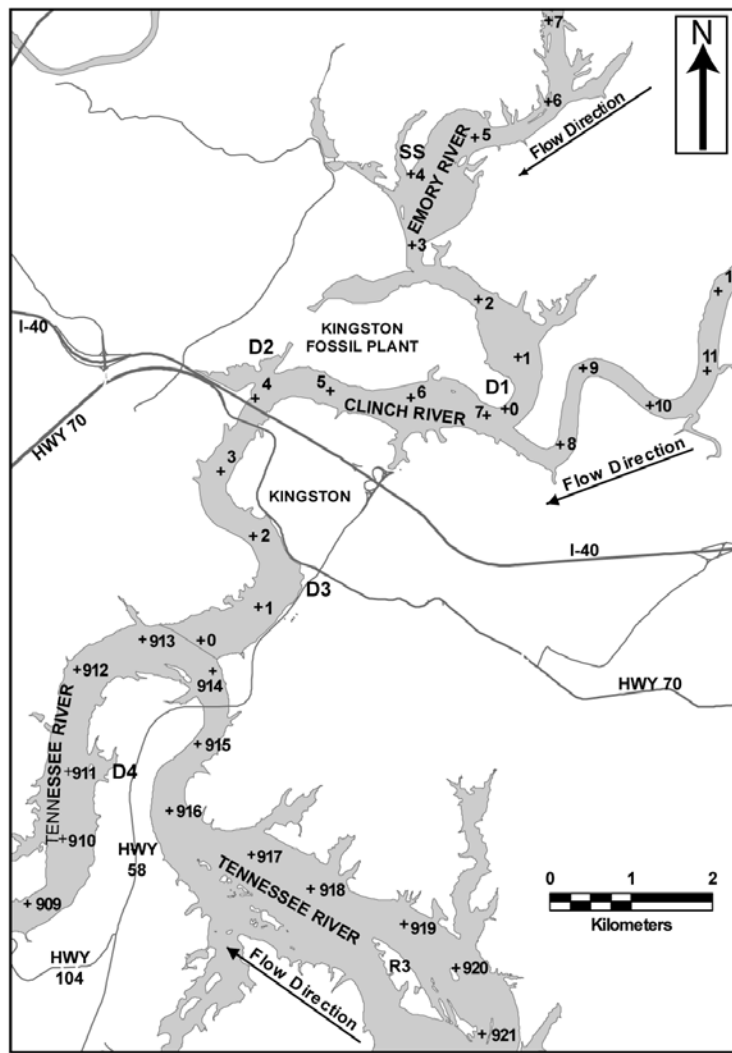
FIGURE 2.

Composition of bolus samples across the study area. All statistical analyses were performed on transformed data but we present untransformed proportions in graphs for clarity. Diptera non-Chironomidae ▨ , Chironomidae ☐ , Hymenoptera ☐ , Hemiptera ▨ , Coleoptera ■ , Other ▨ . We found that location significantly influenced the proportion of the diet that consisted of Chironomidae ($F_{7, 97} = 3.556$, $p = 0.002$) and Hemiptera ($F_{7, 97} = 2.808$, $p = 0.010$). A greater proportion of the diet consisted of Chironomidae at the Spill Site compared to D3 and D4 (all $p \leq 0.031$). A smaller proportion of the diet consisted of Hemiptera at D3 compared to R1 and D1 (all $p \leq 0.039$).
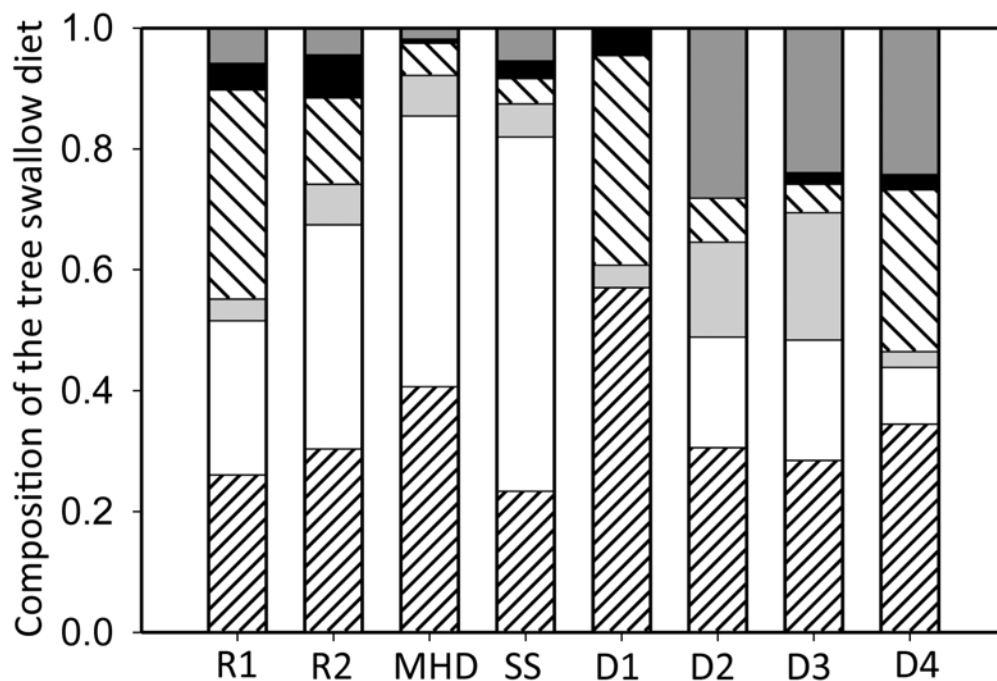
FIGURE 3.

Proportion of the tree swallow diet consisting of insects with an aquatic stage in their life cycle by colony. The proportion of insects with an aquatic stage in their life cycle consumed by tree swallows differed significantly among colonies ($F_{7, 97} = 3.921$, $p = 0.001$). Different letters above bars indicate colonies that were significantly different in post-hoc comparisons ($p < 0.010$).
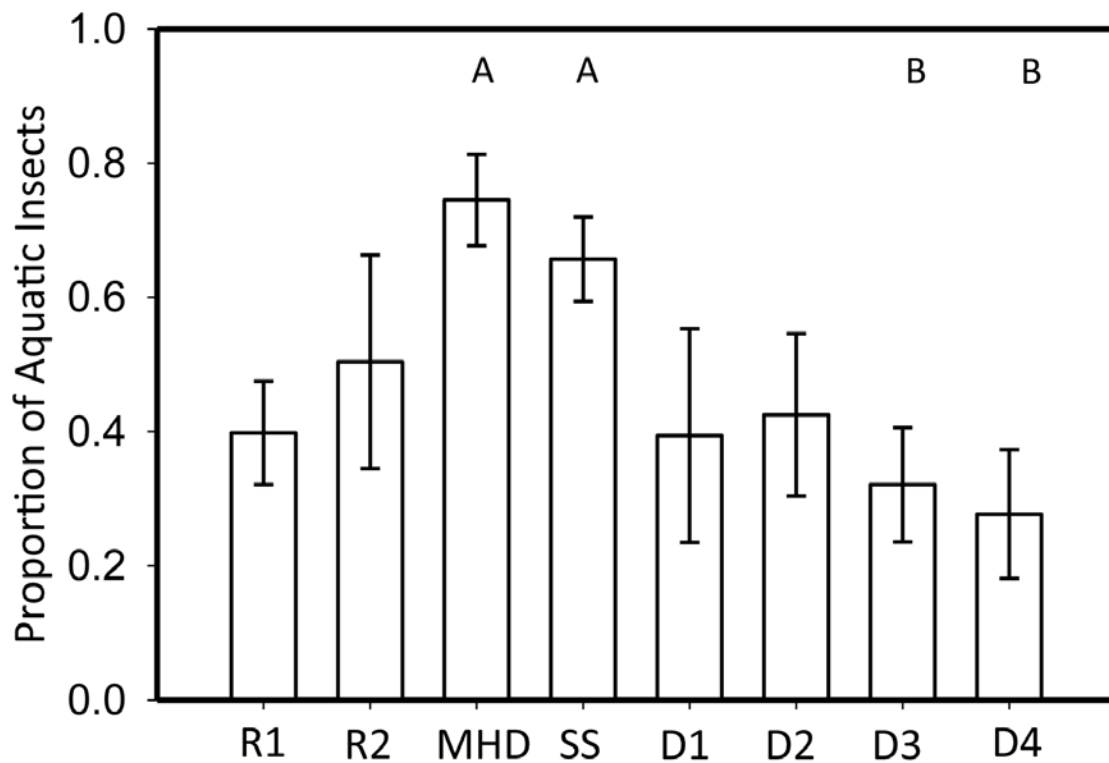
FIGURE 4.

Principal components of trace element concentrations in tree swallow bolus samples by colony PC1 ▢ , PC2 ■ , PC3 ▯ . We found significant differences among colonies in PC1 ($F_{7, 96}$ = 3.359, p = 0.003) and PC3 ($F_{7, 96}$ = 2.977, p = 0.007) but PC2 did not differ among colonies ($F_{7, 96}$ = 1.741, p = 0.109). Letters above bars denote significant differences among colonies for PC1 scores (all $p \leq 0.028$) and letters below bars for PC3 scores (all $p \leq 0.030$).
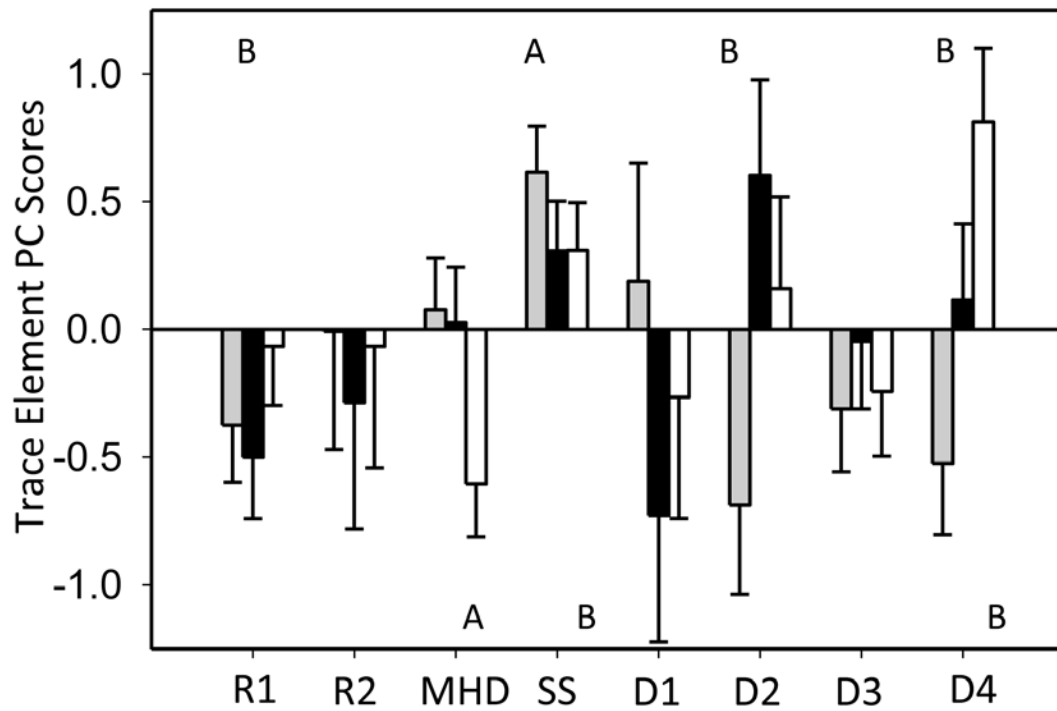
FIGURE 5.

Exposure to trace element contaminants is affected by composition of the tree swallow diet. Tree swallows that consumed a greater proportion of (a) aquatic insects (r^2 = 0.191, $p < 0.001$) and (b) Chironomidae (r^2 = 0.338, $p < 0.001$) were exposed to greater concentrations of trace elements associated with PC1. Consumption of Hemiptera was negatively related to exposure to PC1 contaminants (r^2 = -0.041, p = 0.038). All statistical analyses were performed on transformed data but we present untransformed values in graphs for clarity.
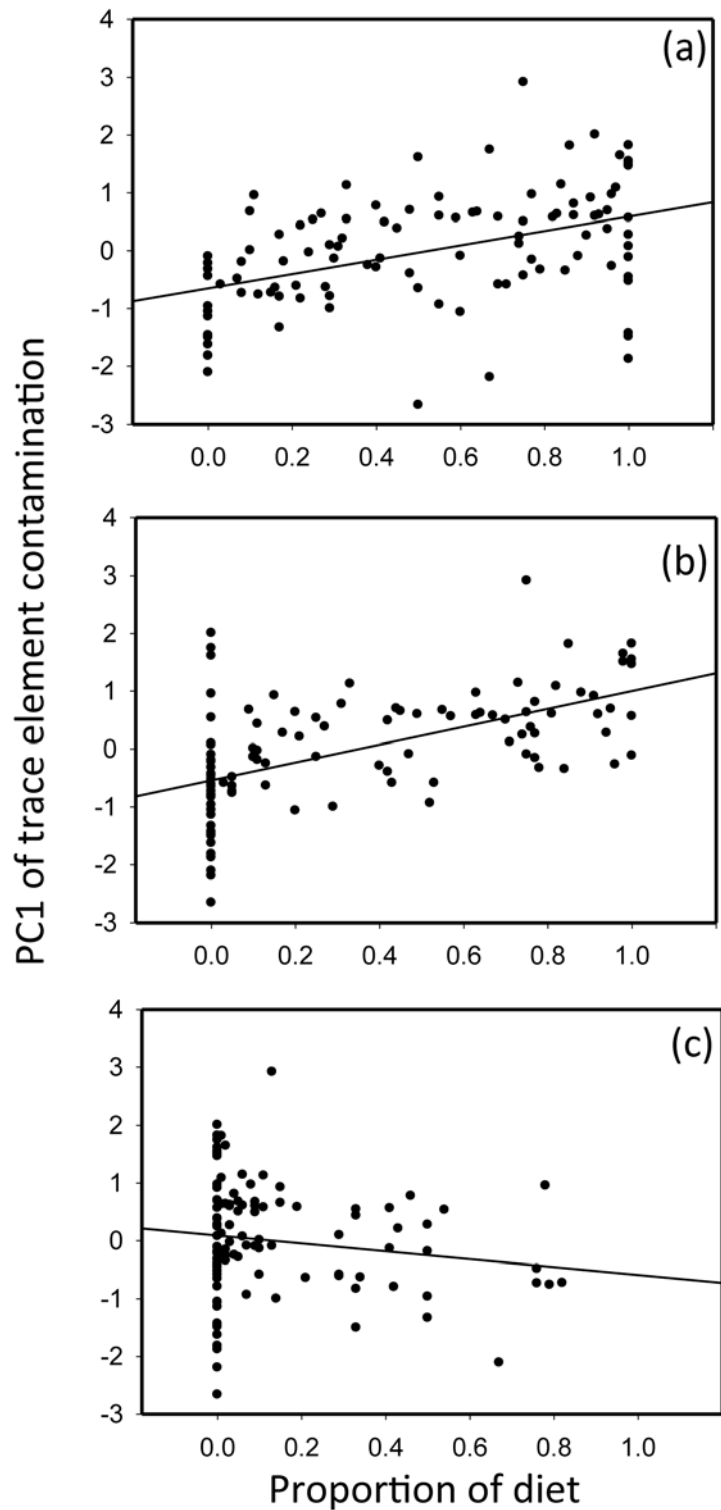
SUPPLEMENTAL TABLE 1.

Description of Tree Swallow colonies located around Kingston, TN in 2011 and 2012.

Colony Name	Location
Reference 1	Ft. Loudoun Dam on the Tennessee River, ≈ 35 km east of Kingston
Reference 2	Tellico Dam on the Little Tennessee River, ≈ 35 km east of Kingston
Reference 3	Long Island on the Tennessee River, 5.5 km upstream from confluence with Clinch River
Melton Hill Dam	On Clinch River, ≈ 35 km east of Kingston, served as positive control
Spill Site	Site of ash spill, on Emory River
Downstream 1	Power Lines cut located near Kingston Fossil Plant, on Emory River at confluence with Clinch River
Downstream 2	Kingston Fossil Plant Discharge Area, on Clinch River, 1.5 km from confluence with Emory River
Downstream 3	Kingston City Park, on Clinch River, 3 km from confluence with Emory River
Downstream 4	Pastures located on Tennessee River, 2.5 km downstream from confluence with Clinch River

SUPPLEMENTAL TABLE 2.

Univariate mean and standard error of trace element concentrations in tree swallow bolus samples by location (μg/mg) for additional elements analyzed in 2011. The concentrations of these elements are not included in PCA analysis because they are analytically problematic, consistently below the detection limit, or are not typically associated with coal-fly ash. The average detection limits were Al, 6.92 μg/mg; Sb, 0.014 μg/mg; Be 0.007 μg/mg; B, 0.69 μg/mg; Co, 0.007 μg/mg; Pb, 0.007 μg/mg; Mo, 0.028 μg/mg; Ni, 0.021 μg/mg ; Ag, 0.007 μg/mg. Aluminum, Sb, and Be were below the detection limit in over half of the samples from at least three of the colonies and were not analyzed further.

E	Reference 1	Reference 2	Melton Hill Dam	Spill Site	Downstream 1	Downstream 2	Downstream 3	Downstream 4
B	3.7 ± 1.5	5.2 ± 3.2	4.7 ± 1.4	3.3 ± 1.2	3.6 ± 3.2	2.4 ± 2.4	1.1 ± 1.7	4.8 ± 1.9
Co	0.05± 0.03	0.05 ± 0.07	0.05 ± 0.03	0.13± 0.03	0.05± 0.07	0.12± 0.05	0.17± 0.04	0.13± 0.04
Pb	0.08± 0.21	0.06 ± 0.44	0.09 ± 0.19	0.10 ± 0.17	0.64 ± 0. 44	0.04 ± 0.33	0.68 ± 0. 23	0.06 ± 0.26
Mo	0.41 ± 0.12	0.33 ± 0.24	0.45 ± 0.11	0.39 ± 0.09	0.41 ± 0.24	0.20 ± 0.18	0.52 ± 0.13	0.23 ± 0.15
Ni	0.51 ± 0.26	0.51 ± 0.53	0.26 ± 0.23	0.58± 0.20	1.25± 0.53	0.83 ± 0.40	0.17 ± 0.28	0.36 ± 0.32
Ag	0.07 ± 0.06	0.17 ± 0.08	0.43 ± 0.06	0.23 ± 0.05	0.30n± 0.013	0.05 ± 0.10	0.09 ± 0.07	0.17 ± 0.08

INTERSPECIFIC DIFFERENCES IN EGG PRODUCTION AFFECT EGG TRACE ELEMENT CONCENTRATIONS AFTER A COAL FLY-ASH SPILL

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INTRODUCTION

Maternal transfer is a significant source of exposure to bioaccumulative contaminants in oviparous vertebrates.[1-3] Maternally transferred contaminants can affect reproductive success by reducing hatching success [4-6] and by inducing developmental malformations that render offspring unviable.[6-9] However, substantial interspecific variation in reproductive traits like clutch size, egg size, egg production synchronicity and rate, and frequency of reproduction could result in among-taxa differences in maternal transfer and reproductive effects that have been relatively unexplored. Furthermore, studies of maternal transfer often examine the contaminant contents of single eggs,[10-15] or pooled egg samples,[16] and assume they are representative of the entire clutch. Studies demonstrating laying order effects on egg contaminant concentrations in birds suggest that this assumption is not always valid,[17-19] but no studies have assessed within-clutch variability in egg contaminant concentrations among species at a single site. Because maternally transferred contaminants can strongly impact reproductive success, understanding how among-species differences in egg production affect embryonic exposure is ultimately critical for assessing health risks in vertebrate assemblages exposed to bioaccumulative contaminants.

Among oviparous amniote vertebrates (birds and most reptiles), contaminants are maternally transferred during egg production, which proceeds in three stages: vitellogenesis (yolk production), albumin deposition, and eggshell formation. During vitellogenesis, phospholipoprotein yolk precursors are produced in the liver, shuttled via the bloodstream to the ovary, and deposited within ovarian follicles prior to ovulation.[20] Because yolk is a complex of protein, lipid, and inorganic nutrients (e.g., Ca, Mg, P, etc.),[20] vitellogenesis may be a route of maternal transfer for contaminants that incorporate into amino acids,[21] fatty acids,[22] or replace inorganic ions.[23] In addition, yolk provides the vast majority of nutrition available for embryogenesis,[20] and as a result is likely to be the primary route of maternal transfer for these contaminants.[24, 25] After ovulation and fertilization, albumin and eggshell are secreted around the ova in consecutive layers by the oviduct.[24-26] Albumin is a proteinaceous complex that can be an additional source of amino acids during embryogenesis,[27] so may also be a route of maternal transfer of contaminants that incorporate into amino acids.[28] Eggshell can be a major source of Ca and Mg during embryogenesis in some species,[25, 29] so may also be a route of maternal transfer of contaminants with similar chemical properties to these inorganic ions.[23]

Whereas the molecular mechanisms of egg production are largely conserved among oviparous amniotes,[20, 30] the synchronicity and rate of egg production vary enormously, especially between birds and turtles. Furthermore, nutrients allocated to egg production may be derived from either recently-assimilated from food (*income*) or mobilized from long-term storage (*capital*), the proportions of which may differ among species[31, 32.32, 33] Turtles provision ovarian follicles synchronously over 3-5 months [33, 34] and ovulate all eggs within a clutch together.[35] Because turtles often feed throughout vitellogenesis but also maintain large fat stores,[36] they likely provision eggs with nutrients derived from both income and capital sources.[37] After ovulation, albumin and eggshell are deposited on all eggs simultaneously in turtles, often over days or even weeks.[38, 39] Utilization of both income and capital resources, in conjunction with a very long egg production cycle, suggests that egg contaminant concentrations should reflect a long-term integration of maternal dietary exposure to contaminants. In contrast, birds establish a follicular hierarchy during vitellogenesis; yolk is deposited in follicles in a rapid and sequentially overlapping manner and ova are ovulated sequentially as they mature, usually within days of recruitment.[31, 40-42] In addition, many birds primarily provision income resources, and very little capital, to egg production.[43] Birds deposit albumin and eggshell on each egg individually and oviposit one egg at a time, usually within 24 hours of ovulation.[40, 41] The extremely short period of egg production in birds suggests that each egg should represent a short-term snapshot of income resources, with capital sources being of less importance to egg formation.[42]

The differences in egg production synchronicity and rate between birds and turtles should result in differences in maternal transfer of contaminants. If birds vary their dietary concentrations of contaminants during egg production, their sequential pattern of egg production should result in variable contaminant concentrations among eggs. In contrast, the synchronous and extended pattern of egg production used by turtles means each egg should exhibit similar contaminant concentrations, regardless of daily differences in contaminant exposure. In addition, migratory birds are likely not exposed to the same contaminants at both wintering and breeding sites, and so may not accumulate as many trace elements as do turtles that live in a contaminated site throughout the year. Taken together, these lines of logic led us to hypothesize that in turtle and bird species with similar dietary compositions, egg contaminant concentrations should be significantly more variable in bird clutches than in turtle clutches, and mean contaminant concentrations should be significantly higher in turtle eggs than in bird eggs. We tested these hypotheses by comparing both contaminant concentrations in eggs and within-clutch variability of contaminant concentrations between tree swallows (*Tachycineta bicolor*) and stinkpot turtles (*Sternotherus odoratus*) inhabiting an area contaminated by trace elements from a recently remediated coal fly-ash spill.

METHODS

FIELD SITE DESCRIPTION

We examined how differences in egg production affected trace element variability in tree swallow and stinkpot eggs at the site of the 2008 Kingston, TN, USA coal fly ash spill. In December, 2008, 4.12 million cubic meters of coal fly-ash were accidentally discharged into the Emory River by the Tennessee Valley Authority's Kingston Fossil Plant.[44] Following the spill, ash was swept downstream to the Clinch River, and eventually was detected in the Tennessee River over 10 km downstream of the spill. Remediation efforts removed the majority of ash by May 2010 (~ 1 year prior to our study) but 400,000 cubic meters of ash remains in the system [45] and ash-derived contaminants may still be entering local food webs. Coal fly-ash contains elevated concentrations of many trace elements, including arsenic (As), barium (Ba), cadmium (Cd), selenium (Se), strontium (Sr), thallium (Tl), and vanadium (V).[46] We co-located our collection efforts for both species in the Emory River within 4 km of the spill to reduce any effects of spatial variability in exposure. All collections occurred in spring, 2011; approximately ~ 2.5 years after the spill event in 2008.

STUDY SPECIES

Tree swallows, *Tachycineta bicolor*, are one of the primary model species used to address the movement of contaminants from aquatic to terrestrial ecosystems.[47] Tree swallows are secondary cavity nesters and readily use nest boxes.[48] Both sexes remain close to their nest site throughout the breeding season and typically forage within 300-500 m of their box[49, 50, 50, 51] They are aerial insectivores and one of their primary food sources when breeding in riparian areas are emerging aquatic insects,[51] making them susceptible to exposure to trace elements originating from aquatic food webs.

Stinkpot turtles, *Sternotherus odoratus*, share several traits with tree swallows that make between-species comparisons of contaminant effects feasible. Stinkpots have small home ranges and long lifespans, and can be particularly susceptible to accumulating contaminants at impacted sites.[52] Stinkpots forage primarily on aquatic invertebrates, including emerging insects,[53] and are thus susceptible to exposure to trace elements in aquatic food

webs. Stinkpots are also easily trapped and densely populated in many aquatic habitats, which makes them easy to sample.

TREE SWALLOW EGG COLLECTION

We installed *T. bicolor* nest boxes in March 2011. Tree swallows are migratory and were present in the area in early March; nesting activity began in mid-April. We checked nest boxes every four days for signs of nest building and clutch initiation. If a nest contained a single egg, we numbered the egg using a non-toxic marker and returned to the box daily to mark new eggs until day 6 of egg-laying. Tree swallows initiating nests in late-April typically lay 5-6 eggs per clutch so we collected all of the eggs on day 6. Female tree swallows begin incubating after laying the penultimate egg,[48] so eggs from 5-egg clutches may have undergone some early embryonic development prior to collection. Because our analysis used whole egg contents (see below), this limited amount of embryonic development is unlikely to have affected the results. Collected eggs were bagged individually and transported to the lab in padded containers. We weighed each egg with a digital balance to the nearest 0.01 g and measured its length and width with digital calipers to the nearest 0.01 mm. Eggs were returned to the collection bag and frozen at -20°C until being prepared for trace element analysis.

STINKPOT EGG COLLECTION

From May-June 2011, we captured gravid stinkpots using baited hoop traps. We removed turtles from traps daily and placed turtles in water-filled plastic tubs and transported them back to a field laboratory. In the laboratory, we palpated female turtles to determine their reproductive state. Of the 32 gravid females captured, we randomly selected 11 gravid females for this study. We weighed each female, and then induced oviposition by injecting them with 20 mg/kg of oxytocin dissolved in deionized water.[54, 55] We placed injected females in plastic tubs with ~2 cm of dechlorinated water, [54] and placed the tubs in a dark room at ~25° C. We checked these females for deposited eggs every two hours. When eggs were present, we gently dried them, weighed them, and measured their length and diameter. We could not determine laying order because multiple eggs were often laid between checks. Every egg from each clutch was then frozen at -20 °C. We palpated females to ensure they had deposited all eggs in their clutches, and then released them at the site of capture the day after completing oviposition.

EGG PREPARATION

Prior to trace element analysis, we removed eggshells from all eggs. We thawed the egg slightly at room temperature and then cracked the eggshell with a scalpel. We then removed the shell and deposited the still-frozen yolk and albumin into autoclaved plastic scintillation vials. We cleaned scalpel blades with Citranox® and rinsed them with Millipore®-filtered water after each egg. We then allowed egg contents to thaw at room temperature. Once thawed, we homogenized egg contents with Teflon spatulas and by vortexing with Teflon beads. We lyophilized egg contents in a freeze drier until they reached asymptotic mass, and stored dried homogenized eggs in a dessicator. We then transferred at least 50 mg into eppendorf tubes for shipment to Dartmouth College for trace elements analysis.

TRACE ELEMENTS ANALYSIS

Preliminary analyses showed that only Ba, Se, Sr, and Tl concentrations were significantly elevated in stinkpot and tree swallow eggs as a result of the coal ash spill compared to reference locations (Hopkins et al. *unpublished data*), so all other trace elements were dropped from further analysis for the purposes of this study. Barium, Se, Sr, and Tl concentrations in eggs were quantified using Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) at the Trace Element Analysis Core at Dartmouth College. Dried eggs were frozen and stored at -20°C prior to sample preparation and analysis. Each egg sample was weighed in a pre-weighed VWR trace metal clean polypropylene centrifuge tube and 0.5 ml of 9:1 HNO_3:HCl (Optima Grade, Fisher Scientific) was added. Individual egg subsample weights were variable but were generally ≈ 0.03g. Egg samples were prepared for acid digestion in batches of 100 samples along with five each of blank, certified reference material, and fortified blank quality control samples. We also digested and analyzed matrix duplicates and matrix duplicate spikes at a frequency of one each per 20 samples. All tubes were lightly capped and placed into a CEM MARS Express (Mathews, NC) microwave digestion unit for an open vessel digestion. A fiber optic temperature probe was placed into one of the sample tubes to provide temperature feedback to the MARS unit and the samples were heated to 95 °C with a ramp to temperature of 15 minutes and held at temperature for 45 minutes. The samples were then allowed to cool and 0.1 ml of H_2O_2 (Optima Grade, Fisher Scientific) was added and the samples were taken through a further microwave heating program. The samples were then brought up to 10 ml with deionized water (Element QPod ®, Millipore, Billarica, MA). All measurements were recorded gravimetrically.

Digested samples were analyzed for Ba, Se, Sr, and Tl by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Concentrations of Ba, Sr, Tl, and Se were measured in He collision mode (4.5 ml min^{-1}) and Selenium (78) was also measured in hydrogen mode (2.5 ml min^{-1}). Analytical procedures followed the general protocols outlined in EPA 6020A; the instrument was calibrated with NIST-traceable standards (DORM-2) and calibration was verified with a second source-traceable standard (NIST Oyster Tissue). Quality control measures included running one empty, metal-free falcon tube as a blank for every twenty samples. Detection limits for each egg sample varied because the mass of each egg sample used in the analysis varied, but all of the samples used in this study were above detection limits for the elements reported in this paper.

Average recovery of Se, Sr and Ba in NIST Oyster Tissue (n = 14) was 109 ±11%, 96 ±5%, 83 ±6%, respectively and average recovery for Se in DORM2 (n = 10) was 111 ±13%. Thallium was not certified in either reference material. Average recoveries of fortified blanks (n = 25) taken through the digestion and analysis procedure were 103%, 105%, 103 %, 99%, for Se, Sr, Ba, Tl respectively. Average relative percent difference of digestion duplicates (n = 25) were 17%, 13%, 20 %, 12%, for Se, Sr, Ba, Tl respectively. Average recoveries of digestion spiked samples were 114%, 109%, 110% and 96% for Se, Sr, Ba, Tl respectively. Average relative percent difference of analysis duplicates (n = 25) were 8%, 5%, 3 %, 7%, for Se, Sr, Ba, Tl respectively. Average recoveries of analysis spiked samples were 109%. 113%, 114% and 91% for Se, Sr, Ba, Tl respectively.

STATISTICAL ANALYSIS

We compared egg concentrations of Ba, Se, Sr, and Tl between stinkpots and tree swallows using MANOVA, with maternal ID as a random effect to account for clutch effects. We compared egg trace element concentration variability directly by calculating within-clutch unbiased coefficients of variation (COV)[56] for each trace element in each clutch. Thus, clutch was the experimental unit in our analysis. Following Sokal and Braumann, we log-

transformed each COV to normalize the data, and compared mean log-COV of each trace element between stinkpots and tree swallows using a MANOVA.[57]

We also calculated repeatability for each trace element in both Stinkpots and Tree Swallow clutches. Repeatability (R) is commonly used in quantitative genetics as an estimate of the proportion of variance in a trait that is attributable to differences between rather than within individuals,[58-60] and has recently been used to examine changes in variability of blood trace element concentrations in arctic birds,[61] and to examine reference material consistency.[62] Therefore, R should indicate whether the trace element concentration of a single egg is representative of the concentrations of all eggs within a clutch. Briefly, R can be expressed as the intraclass correlation coefficient (ICC): [56]

$$R = \frac{S_a^2}{S^2 + S_a^2}$$

where S_a^2 is the among-groups variance component, and S^2 is the within-groups variance component. We followed Hendricks [59] in deriving species-specific R values for each trace element from one-way random-effects ANOVAs run in SAS 9.3 (SAS Corporation, Cary NC). We also estimated R using a more recent linear mixed-models (LMM) approach in PROC MIXED following Nakagawa and Schielzeth [63] but the results were not quantitatively different from the one-way ANOVAs, likely because of the relative simplicity of our statistical models. We then calculated standard errors for R following Becker [60] in Excel (Microsoft, Redmond WA). We used one-tailed F-tests to determine whether R was significantly greater than zero for each trace element within both Stinkpots and Tree Swallows. Finally, we compared trace element R values between species using two-tailed F-tests calculated following McGraw and Wong.[64]

Because tree swallow eggs were collected in order of laying, we were also able to determine whether laying order affected within clutch variability in Ba, Se, Sr, and Tl concentrations, as well as egg mass and volume. We could not investigate laying order effects in stinkpots because precise laying sequence was not known in most cases. We used repeated-measures ANOVA and post-hoc Tukey Tests to examine changes in trace element concentrations in the first five eggs laid in each tree swallow clutch. We omitted the sixth egg because we had an insufficient sample size of six egg clutches (n = 4). If the data violated the assumption of sphericity (that variances of the differences among related groups are equal), we used the Greenhouse-Geisser correction to reduce the chances of committing a Type 1 error. We also ranked egg trace element concentrations from lowest concentration to highest for visual comparison.

In all statistical tests, we assessed univariate normality and homoscedasticity of variance using Levene's Tests, Shapiro-Wilk Tests, and normal probability plots. Sample sizes were not large enough to test for violations of multivariate normality, so we used Pillai's Trace as a test statistic in all multivariate analyses because it is the most robust to violations of multivariate normality.[65] We followed MANOVAs with post-hoc univariate ANOVAS to examine within-element differences between species. We judged two-way statistical tests at $\alpha = 0.05$ and one-way statistical tests at $\alpha = 0.025$, and presented means ± 1 SE, unless otherwise stated.

RESULTS

Stinkpot clutch sizes averaged 3.36 ± 0.31 (range 2-5 eggs) while tree swallow clutch sizes averaged 5.42 ± 0.19 (range 4-6 eggs). Total clutch mass in stinkpots averaged 15.62 ± 1.52 (range 9.56-24.33 g), while tree swallow total clutch mass averaged 9.88 ± 0.343 g (range 8.20-11.29 g). Mean egg trace element concentrations differed significantly between stinkpots and tree swallows (Fig. 1; Pillai's Trace = 3.543, $F_{88, 316} = 27.82$, $p < 0.001$). Post-hoc

univariate ANOVAs showed that Ba, Se, Sr, and Tl concentrations were all significantly higher in stinkpot eggs than in tree swallow eggs (Ba: $F_{22,79}$ = 70.39, p < 0.001; Se: $F_{22,79}$ = 34.57, p < 0.001; Sr: $F_{22,79}$ = 70.26, p < 0.001; Tl: $F_{22,79}$ = 419.38, p < 0.001). Mean clutch coefficients of variation (COV) also differed significantly between species (Fig. 2; Pillai's Trace = 0.922, $F_{4, 18}$ = 53.38, p < 0.001), and post-hoc univariate ANOVAs showed that the COV of all four elements were significantly higher in tree swallow clutches than in stinkpot clutches (Ba: $F_{1,22}$ = 196.59, p < 0.001; Se: $F_{1,22}$ = 6.48, p = 0.019; Sr: $F_{1,22}$ = 18.22, p < 0.001; Tl: $F_{1,22}$ = 14.68, p = 0.001). Within-clutch repeatabilities (R) were significantly greater than zero in all species-element combinations except Ba in tree swallows (Table 1). Within-clutch R of all four elements were also significantly higher in stinkpots than in tree swallows (Fig. 3; Ba: $F_{10,11}$ = 43.29, p < 0.001; Se: $F_{10,11}$ = 12.20, p < 0.001; Sr: $F_{10,11}$ = 4.80, p = 0.008; Tl: $F_{10,11}$ = 5.10, p = 0.006).

Mean Ba, Se, and Sr concentrations changed with laying order in tree swallow eggs (Fig 4). Barium concentrations increased with laying order ($F_{2, 13}$ = 65.281, p < 0.001) and the fifth egg laid had significantly higher Ba concentrations than the first two eggs (both, p < 0.001) and was nearly significantly greater than the third egg (p = 0.087). Likewise, Sr concentrations increased with laying order ($F_{2, 12}$ = 13.478, p = 0.001) and the fifth laid egg had significantly higher Sr concentrations than the first three eggs of the clutch (all $p \leq$ 0.034). In contrast, Se concentrations were significantly higher early in the laying order ($F_{4, 32}$ = 19.275, p < 0.001) and pairwise comparisons indicated that the first laid egg had significantly higher Se concentrations than all subsequently laid eggs (all $p \leq$ 0.014) and that the second laid egg had significantly greater concentrations of Se than the fourth laid egg (p = 0.009). Thallium concentrations did not change with laying order in tree swallows (Figure 4, $F_{4, 32}$ = 2.301, p = 0.08). Mean ranks suggested similar effects of laying order and corrected for among clutch differences in trace element concentrations in eggs. Barium and Sr concentration rank increased consecutively, while Se concentration rank decreased from egg 1 to egg 4, and Tl concentration rank did not change among eggs (Figure 4). Egg mass increased significantly with laying order ($F_{4, 32}$ = 13.478, p = 0.001) and the first two eggs laid were significantly lighter than eggs 4 and 5 (Figure 5A; all $p \leq$ 0.004). We detected a similar pattern for egg volume ($F_{2, 14}$ = 8.846, p = 0.005) though the first two eggs in the clutch were only significantly lower in volume than the fifth egg (Figure 5B; both $p \leq$ 0.004).

DISCUSSION

Our study demonstrated that trace element concentrations are significantly more variable within clutches of bird eggs than in turtle eggs collected from the site of a recently-remediated coal fly-ash spill, and that the variability differs among elements measured. We found that contaminant concentrations were significantly higher in turtle eggs than in bird eggs, and that laying order effects differed among trace elements in birds. However, the concentrations of Ba, Se, Sr, and Tl in both stinkpot and tree swallow eggs were below known toxic thresholds (data from birds)[66, 67] so even the highest concentrations we observed are unlikely to have developmental consequences. Indeed, other studies have detected limited effects of trace element exposure on reproductive success of tree swallows (Chapter 1) and turtles in this system. These results highlight how differences in egg production synchronicity and rate can result in differences in maternal transfer between species even though both species utilize homologous physiological mechanisms of egg production and feed at least to some degree on similar diets.

Mechanisms of maternal transfer should be conserved in stinkpots and tree swallows due to their homologous physiological mechanisms of egg production. However, mechanisms of maternal transfer likely differ among the trace elements we studied, thus there was no *a priori* reason to expect that different trace elements should exhibit similar patterns of concentration variability. Barium and Sr can both substitute for Ca, so both are likely to be

incorporated into yolk precursors instead of Ca during vitellogenesis.[29, 68] Selenium can substitute for S in cysteine and methionine,[69, 70] both of which have been isolated from egg yolk and albumin of chicken eggs [27, 71, 72] and this is likely how Se enters stinkpot and tree swallow eggs. Thallium has not been studied as extensively as other trace elements, but is known to bind to sulfhydryl groups of cysteine and methionine,[73] and thus may also be transferred to eggs in yolk and albumin. Elemental Tl is also easily absorbed across biological membranes,[9] and so might be passively absorbed into eggs at any point prior to oviposition.

EGG VARIABILITY

Barium, Se, Sr, and Tl were significantly more variable and less repeatable in tree swallow eggs than in stinkpot eggs. Despite species differences, repeatabilities of all elements except Ba in Tree Swallow eggs were significantly greater than zero. Taken together, these results support our hypothesis that sequential egg production in birds results in greater within-clutch variation in trace element concentration than synchronous egg production in turtles. Because tree swallows can fly between contaminated and uncontaminated sites and forage on both contaminated and uncontaminated prey during egg production, the relative amounts of trace elements maternally transferred to different eggs likely differ on a daily basis.[76, 77] In contrast, stinkpots produce all eggs in a clutch synchronously,[33, 34] thus all eggs should receive similar amounts of contaminants throughout egg production.

LAYING ORDER

In tree swallows, within-clutch variability manifested in significant laying order effects in Ba, Se, and Sr concentrations, but not in Tl concentrations. Barium and Sr concentrations increased with laying order, while Se decreased with laying order. The coincident increase of Ba and Sr with laying order may be because both elements can replace Ca during vitellogenesis.[29, 68] In other species, Se is incorporated into proteins in both yolk and albumin [74] while minerals like Ca, and hence Ba and Sr, are incorporated into yolk. This could contribute to the laying order effect we detected if the proportions of yolk and albumin change with laying order, which has been suggested for tree swallows.[42] Thallium concentrations were low in all tree swallow eggs and this may have inhibited our ability to detect a laying order effect if one existed. Other studies have suggested that laying order effects were more difficult to detect at low contaminant concentrations [75-77] and a study on ducks reported more pronounced laying order effects at higher trace element concentrations.[78] Other studies that have examined the effects of laying order on the deposition of Se and other trace elements in avian eggs have found that trace elements may increase,[47] decrease,[47, 79] or not change with laying order.[17-19, 76, 80, 81] The inconsistency of laying order effects among studies may be due to differential availability of contaminants during egg production,[17, 82, 83] differences among contaminants in how they are incorporated into maternal tissue and eggs,[84] and/or interactions among co-occurring contaminants.[18, 85] Other studies indicated that small clutch sizes seem more likely to produce a laying order effect than large clutches.[47, 75, 77] When and if stored resources are utilized in egg production may also affect contaminant deposition and produce laying order effects.[84] Methodological differences among studies may have contributed as well, because some of these studies inferred laying order or only sampled the laying order in a portion of the clutch.[80, 82] Many of the differences we detected only occurred between the first eggs and the last eggs laid and would have been missed had we examined only a portion of the clutch.

Egg mass and volume increased with laying order in our population of tree swallows as has been found in other populations,[42, 86] but see Whittingham et al.[87] One of these studies found that later-laid eggs contained larger yolks; though changes in albumin with laying order were not addressed.[44] An increase in yolk mass could contribute

to the observed increase in Ba and Sr concentrations with laying order if concentrations of minerals increased concomitantly with yolk size. While Se is incorporated into yolk proteins, it is also found extensively in the albumin.[74] If increased yolk size is associated with a reduction in the amount of albumin in the egg, that could lead to the observed reduction in Se with laying order that we detected.

MEAN CLUTCH CONCENTRATIONS

In addition to differences in clutch variability, we found that Ba, Se, Sr, and Tl concentrations were all significantly higher in stinkpot eggs than in tree swallow eggs. Because vitellogenesis in particular, and egg production in general, occurs over multiple months in turtles,[35, 36] it is possible that turtles maternally transfer more contaminants to their eggs simply because they consume and assimilate more contaminants during egg production than do tree swallows. In this population, tree swallows also rely on terrestrial uncontaminated prey for ~35-65% of their diet at these colonies (Beck et al., *In Press*), which further suggests that tree swallows may not consume as many contaminants during egg production as do stinkpots. Furthermore, as small songbirds, tree swallows likely do not rely heavily on capital resources for reproduction,[17, 42] and so should maternally transfer fewer previously-bioaccumulated contaminants from long-term storage than do turtles. For migratory birds like tree swallows, this pattern of egg formation likely limits the sources of contaminants in their eggs to local origin during the nesting season. In contrast, turtles rely heavily on capital resources during vitellogenesis,[36] and so likely maternally transfer previously-bioaccumulated contaminants from long-term storage in addition to contaminants recently assimilated from diet.

CONCLUSIONS

Our study suggests that sequential egg production in birds results in greater within-clutch variation in contaminant concentrations than does synchronous egg production in turtles, and that long-term egg production in turtles may contribute to higher mean egg contaminant concentrations than does rapid egg production in birds. From a purely practical perspective, our results indicate that contaminant concentrations in a single egg are more likely to be representative of the mean contaminant concentrations of the entire clutch in turtles than in birds. As a result, bird embryos may experience somewhat variable exposure to contaminants within a clutch, while turtle embryos will experience relatively similar exposure within a clutch. These differences may be especially pronounced in systems where turtles' entire home ranges are contaminated and they can only forage on contaminated prey, while birds can fly between and forage in both contaminated and uncontaminated areas. Furthermore, our results suggest that contaminant effects on reproduction in oviparous vertebrates may not be generalizable among species that differ in reproductive phenology. Further experimentation is needed to determine whether the species differences in maternal transfer we observed result in differential risks of adverse effects to developing embryos.

REFERENCES

(1) Brasso, R. L.; Abdel Latif, M. K.; Cristol, D. A., Relationship between laying sequence and mercury concentration in tree swallow eggs. Environ. Toxicol. Chem. 2010, *29*, (5), 1155-1159.

(2) Nagle, R. D.; Rowe, C. L.; Congdon, J. D., Accumulation and selective maternal transfer of contaminants in the turtle *Trachemys scripta* associated with coal ash deposition. Arch. Environ. Contam. Toxicol. 2001, *40*, 531-536.

(3) Hopkins, W. A.; DuRant, S. E.; Staub, B. P.; Rowe, C. L.; Jackson, B. P., Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. Environ. Health Perspect. 2006, *114*, (5), 661-666.

(4) Ohlendorf, H. M.; Hoffman, D. J.; Saiki, M. K.; Aldrich, T. W., Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. Sci. Total Environ. 1986, *52*, 49-63.

 (5) Smith, G. J.; Anders, V. P., Toxic effects of boron on mallard reproduction. Environ. Toxicol. Chem. 2009, *8*, (10), 943-950.

(6) Domingo, J. L., Vanadium: a review of the reproductive and developmental toxicity. Reproductive Toxicology Review 1996, *10*, (3), 175-182.

(7) Hoffman, D. J.; Heinz, G. H., Embryotoxic and teratogenic effects of selenium in the diet of mallards. Journal of Toxicology and Environmental Health 1988, *24*, (4), 477-490.

(8) Leonard, A.; Lauwerys, R. R., Carcinogenicity, teratogenicity, and metagenicity of arsenic. Mutation Research/Revies in Genetic Toxicology 1980, *75*, (1), 49-62.

(9) John Peter, A. L.; Viraraghavan, T., Thallium: a review of public health and environmental concerns. *Environ. Int.* 2005, *31*, (4), 493-501.

(10) Guirlet, E.; Das, K.; Girondot, M., Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea*) of French Guiana. *Aquat. Toxicol. (Amst.)* 2008, *88*, 267-276.

(11) Burger, J.; Gibbons, J. W., Trace elements in egg contents and egg shells of slider turtles (*Trachemys scripta*) from the Savannah River Site. Arch. Environ. Contam. Toxicol. 1998, *34*, (4), 382-386.

(12) Nyholm, N. E. I., Influence of heavy metal exposure during different phases of the ontogeny on the development of Pied Flycatchers, *Ficedula hypoleuca*, in natural populations. Arch. Environ. Contam. Toxicol. 1998, *35*, (4), 632-637.

(13) Burger, J.; Gochfeld, M., Heavy metal and selenium concentrations in eggs of herring gulls (*Larus argentatus*): temporal differences from 1989 to 1994. Arch. Environ. Contam. Toxicol. 1995, *29*, (2), 192-197.

(14) Fernie, K. J.; Bortolotti, G. R.; Smits, J. E.; Wilson, J.; Drouillard, K. G.; Bird, D. M., In egg composition of American Kestrels to dietary polychlorinated biphenyls. J. Toxicol. Environ. Health Part A 2000, *60*, (4), 291-303.

(15) Mora, M. A., Organochlorines and trace elements in four colonial waterbird species nesting in the lower Laguna Madre, Texas. Arch. Environ. Contam. Toxicol. 1996, *31*, 533-537.

(16) Bishop, C. A.; Brown, G. P.; Brooks, R. J.; Lean, D. R. S.; Carey, J. H., Organochlorine contaminant concentrations in eggs and their relationship to body size, and clutch characteristics of the female Common Snapping Turtle (*Chelydra serpentina serpentina*) in Lake Ontario, Canada. Arch. Environ. Contam. Toxicol. 1994, *27*, 82-87.

(17) Heinz, G. H., Selenium in birds. In Environmental contaminants in wildlife: interpreting tissue concentrations., Beyer, W. N.; Heinz, G. H.; Redmon-Norwood, A. W., Eds. CRC Lewis: Boca Raton, FL, 1996; pp 447-458.

(18) Stanley, T. R., Jr.; Spann, J. W.; Smith, G. J.; Rosscoe, R., Main and interactive effects of arsenic and selenium on mallard reproduction and duckling growth and survival. Arch Environ Contam Toxicol 1994, *26*, (4), 444-451.

(19) Sanpera, C.; Morera, M.; Crespo, S.; Ruiz, X.; Jover, L., Trace Elements in Clutches of Yellow-Legged Gulls, Larus cachinnans, from the Medes Islands, Spain. Bulletin of Environmental Contamination and Toxicology 1997, *59*, (5), 757-762.

 (20) Wallace, R. A., Vitellogenesis and oocyte growth in nonmammalian vertebrates. In Developmental Biology, a Comprehensive Synthesis., Browder, L. W., Ed. Plenum Press: New York, NY, 1985; Vol. 1, pp 127-177.

(21) Unrine, J. M.; Jackson, B. P.; Hopkins, W. A.; Romanek, C. S., Isolation and partial characterization of proteins involved in maternal transfer of selenium in the western fence lizard (*Sceloporus occidentalis*). Environ. Toxicol. Chem. 2006, *25*, (7), 1864-1867.

(22) Rauschenberger, R. H.; Sepulveda, M. S.; Wiebe, J. J.; Szabo, N. J.; Gross, T. S., Predicting maternal body burdens of organochlorine pesticides from eggs and evidence of maternal transfer in Alligator mississippiensis. Environ. Toxicol. Chem. 2009, *23*, (12), 2906-2915.

(23) Mora, M. A., Heavy metals and metalloids in egg contents and eggshells of passerine birds from Arizona. Environ. Pollut. 2003, *125*, 393-400.

(24) Stevens, L., Egg White Proteins. Comp. Biochem. Physiol. B. 1991, *100*, (1), 1-9.

(25) Packard, M. J.; DeMarco, V. G., Eggshell structure and formation in eggs of oviparous reptiles. In Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles, Deeming, D. C.; Ferguson, M. W. J., Eds. Cambridge University Press: UK, 1991; pp 53-69.

(26) Board, R. G.; Sparks, N. H. C., Shell structure and formation in avian eggs. In Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles, Deeming, D. C.; Ferguson, M. W. J., Eds. Cambridge University Press: UK, 1991; pp 71-86.

(27) Bergmann, M.; Niemann, C., On the structure of proteins: cattle hemoglobin, egg albumin, cattle fibrin, and gelatin. J. Biol. Chem. 1937, *118*, 301-314.

(28) Richards, M. P.; Steel, N. C., Trace element metabolism in the developing avian embryo: a review. J. Exp. Zool. 1987, Supplement *1*, 39-51.

(29) Simkiss, K., Calcium metabolism and avian reproduction. Biol. Rev. (Camb*.)* 1961, *36*, 321-367.

(30) Wiley, H. S.; Wallace, R. A., The Structure of Vitellogenin. J. Biol. Chem. 1981, *256*, (16), 8626-8634.

(31) Thomas, V. G., Body condition, ovarian hierarchies, and their relation to egg formation in Anseriform and Galliform species. In Acta XIX Congresses Internationalis Ornithologici, Quellet, H., Ed. National Museum of Natural Science: Ottawa, ON, Canada, 1988; pp 353-363.

(32) Drent, R. H.; Daan, S., The prudent parent: energetic adjustments in avian breeding. Ardea 1980, *68*, 225-252.

(33) Mendonça, M. T., Photothermal effects on the ovarian cycle of the musk turtle, *Sternotherus odoratus*. Herpetologica 1987, *43*, (1), 82-90.

(34) McPherson, R. J.; Boots, L. R.; MacGregor, R., III; Marion, K. R., Plasma steroids associated with seasonal reproductive changes in a multiclutched freshwater turtle, *Sternotherus odoratus*. Gen. Comp. Endocrinol. 1982, *48*, 440-451.

(35) Ewert, M. A., Embryology of turtles. In Biol. Reptil., Gans, C.; Billet, F.; Maderson, P. F. A., Eds. John Wiley and Sons: New York, NY, 1985; Vol. 14, pp 76-267.

(36) Ho, S. M.; Kleis, S.; McPherson, R.; Heisermann, G. J.; Callard, I. P., Regulation of vitellogenesis in reptiles. Herpetologica 1982, *38*, (1), 40-50.

(37) Van Dyke, J. U.; Beaupre, S. J.; Kreider, D. L., Snakes allocate amino acids acquired during vitellogenesis to offspring: are capital and income breeding consequences of variable foraging success? Biol. J. Linn. Soc. 2012, *106*, (2), 390-404.

(38) Tinkle, D. W.; Gibbons, J. W. The distribution and evolution of viviparity in reptiles.; Museum of Zoology, University of Michigan: Ann Arbor, MI, 1977.

(39) Powell, C. B., Female sexual cycles of *Chrysemys picta* and *Clemmys insculpta* in Nova Scotia. Can. Field-Nat. 1967, *81*, 134-140.

(40) Ojanen, M., Egg development and the related nutrient reserve depletion in the pied flycatcher, Ficedula hypoleuca. Ann. Zool. Fenn. 1983, *20*, 293-300.

(41) Etches, R. J.; Petitte, J. N., Reptilian and avian follicular hierarchies: models for the study of ovarian development. J. Exp. Zool. 1990, Supplement *4*, 112-122.

(42) Ardia, D. R.; Wasson, M. F.; Winkler, D. W., Individual quality and feed availability determine yolk and egg mass and egg composition in tree swallows *Tachycineta bicolor*. J. Avian Biol. 2006, *37*, 252-259.

(43) Klaassen, M.; Lindstrom, A.; Meltofte, H.; Piersma, T., Arctic waders are not capital breeders. Nature 2001, *413*, 794.

(44) TVA Corrective Action Plan for the TVA Kingston Fossil PLant Ash Release; Kingston, TN, 2009.

(45) Yankee, D.; McCracken, S.; Zeller, C. In TVA Kingston ash recovery status., Proceedings of the twenty-first Tennessee Water Resources Symposium, 2011; Tennessee Section of the American Water Resources Association: 2011.

(46) Rowe, C. L.; Hopkins, W. A.; Congdon, J. D., Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: A review. Environ. Monit. Assess. 2002, *80*, 207-276.

(47) Custer, C. M.; Gray, B. R.; Custer, T. W., Effects of egg order on organic and inorganic element concentrations and egg characteristics in tree swallows, *Tachycineta bicolor*. Environ Toxicol Chem 2010, *29*, (4), 909-921.

(48) Robertson, R. J.; Stutchbury, B. J.; Cohen, R. R.; Winkler, D. W.; Hallinger, K. K.; Ardia, D. R. Tree Swallow. http://bna.birds.cornell.edu/bna/species/011/articles/introduction (April 10).

(49) Dunn, P. O.; Hannon, S. J., Effects of food abundance and male parental care on reproductive success and monogamy in tree swallows. Auk 1992, *109*, (3), 488-499.

(50) Quinney, T. E.; Ankney, C. D., Prey size selection by tree swallows. *Auk* 1985, *102*, 245-250.

(51) Custer, C. M., Swallows as sentinel species for contaminant exposure and effect studies. In Wildlife Ecotoxicology: Forensic Approaches, JE, E.; CA, M.; CA, B., Eds. Springer: New York, 2011; pp 45-91.

(52) Bergeron, C. M.; Husak, J. F.; Unrine, J. M.; Romanek, C. S.; Hopkins, W. A., Influence of feeding ecology on blood mercury concentrations in four species of turtles. Environ. Toxicol. Chem. 2007, *26*, (8), 1733-1741.

(53) Ernst, C. H.; Lovich, J. E., Turtles of the United States and Canada. 2nd ed.; Johns Hopkins University Press: Baltimore, MD, 2009.

(54) Ewert, M. A.; Legler, J. M., Hormonal induction of oviposition in turtles. Herpetologica 1978, *34*, (3), 314-318.

(55) Tucker, J. K.; Thomas, D. L.; Rose, J., Oxytocin dosage in turtles. Chelonian Conserv. Biol. 2007, 6, (2), 321-324.

(56) Sokal, R. R.; Rohlf, E. J., Biometry: The Principles and Practices of Statistics in Biological Research. 3 ed.; W.H. Freeman: San Francisco, CA, 1994.

(57) Sokal, R. R.; Braumann, C. A., Significance tests for coefficients of variation and variability profiles. Syst. Zool. 1980, 29, 50-66.

(58) Lessells, C. M.; Boag, P. T., Unrepeatabile repeatabilities: a common mistake. Auk 1987, 104, (1), 116-121.

(59) Hendricks, P., Repeatability of size and shape of American Pipit eggs. Can. J. Zool. 1991, 69, 2624-2628.

(60) Becker, W. A., Manual of Quantitative Genetics. Students Book Corporation: Pullman, WA, 1975; p 170.

(61) Wayland, M.; Alisauskas, R. T.; Kellett, D. K.; Mehl, K. R., Trace element concentrations in the blood of nesting King Eiders in the Canadian Arctic. Arch. Environ. Contam. Toxicol. 2008, 55, 683-690.

(62) Sturgeon, R. E.; Willie, S. N.; Yang, L.; Greenberg, R.; Spatz, R. O.; Chen, Z.; Scriver, C.; Clancy, V.; Lam, J. W.; Thorrold, S., Certification of a fish otolith reference material in support of quality assurance for trace element analysis. J. Anal. At. Spectrom. 2005, 20, 1067-1071.

(63) Nakagawa, S.; Schielzeth, H., Repeatability for Gaussian and non-Gaussian data: a practical guide for biologists. Biol. Rev. 2010, 85, 935-956.

(64) McGraw, K. O.; Wong, S. P., Forming inferences about some intraclass correlation coefficients. Psychological Methods 1996, 1, (1), 30-46.

(65) Scheiner, S. M., MANOVA: Multiple response variables and multispecies interactions. In Design and Analysis of Ecological Experiments, Scheiner, S. M.; Gurevitch, J., Eds. Oxford University Press: New York, NY, 2001; pp 99-115.

(66) Janz, D. M.; DeForest, D. K.; Brooks, M. L.; Chapman, P. M.; Gilron, G.; Hoff, D.; Hopkins, W. A.; McIntyre, D. O.; Mebane, C. A.; Palace, V. P.; Skorupa, J. P.; Wayland, M., Selenium toxicity to aquatic organisms. In Ecological Assessment of Selenium in the Aquatic Environment., Chapman, P. M.; Adams, W. J.; Brooks, M. L.; Delos, C. G.; Luoma, S. N.; Maher, W. A.; Ohlendorf, H. M.; Presser, T. S.; Shaw, D. P., Eds. CRC Press: Boca Raton, FL, USA, 2010; pp 141-231.

(67) Ridgway, L. P.; Karnofsky, D. A., The effects of metals on the chick embryo: toxicity and production of abnormalities in development. Ann. N. Y. Acad. Sci. 1952, 55, 203-215.

(68) Simkiss, K., The sources of calcium for the ossification of the embryos of the giant leathery turtle. Comp. Biochem. Physiol. 1962, 7, 71-79.

(69) Unrine, J. M.; Jackson, B. P.; Hopkins, W. A., Selenomethionine biotransformation and incorporation into proteins along a simulated terrestrial food chain. Environ. Sci. Technol. 2007, *41*, 3601-3606.

(70) Maher, W.; Roach, A.; Doblin, M.; Fan, T.; Foster, S.; Garrett, R.; Möller, G.; Oram, L.; Wallschläger, D., Environmental sources, speciation, and partitioning of selenium. In Ecological Assessment of Selenium in the Aquatic Environment, Chapman, P. L.; Adams, W. J.; Brooks, M. L.; Delos, C. G.; Luoma, S. N.; Maher, W. A.; Ohlendorf, H. M.; Presser, T. S.; Shaw, D. P., Eds. CRC Press: Boca Raton, FL, 2010; pp 47-92.

(71) Mecham, D. K.; Olcott, H. S., Phosvitin, the principle phosphoprotein of egg yolk. J. Am. Chem. Soc. 1949, *71*, (11), 3670-3679.

(72) Wahli, W., Evolution and expression of vitellogenin genes. Trends Genet. 1988, *4*, (8), 227-232.

(73) Galvan-Arzate, S.; Santamaria, A., Thallium toxicity. Toxicol. Lett. (Shannon*)* 1998, *99*, (1), 1-13.

(74) Jacobs, K.; Shen, L. H.; Benemariya, H.; Deelstra, H., Selenium Distribution in Egg-White Proteins. Z. Lebensm.-Unters.-Forsch. 1993, 196, (3), 236-238.

(75) Becker, P. H., Egg mercury levels decline with laying sequence in Charadriiformes. Bulletin of Environmental Contamination and Toxicology 1992, *48*, 762-767.

(76) Dauwe, T.; Janssens, E.; Bervoets, L.; Blust, R.; Eens, M., Heavy-metal concentrations in female laying great tits (Parus major)and their clutches. Arch Environ Contam Toxicol 2005, *49*, (2), 249-256.

  (77) Evers, D. C.; Taylor, K. M.; Major, A.; Taylor, R. J.; Poppenga, R. H.; Scheuhammer, A. M., Common loon eggs as as indicators of methylmercruy availability in North America. Ecotoxicology 2003, *12*, 69-81.

(78) Kennamer, R. A.; Stout, J. R.; Jackson, B. P.; Colwell, S. V.; Brisbin, I. L.; Burger, J., Mercury patterns in wood duck eggs from a contaminated reservoir in South Carolina, USA. Environ Toxicol Chem 2005, *24*, (7), 1793-1800.

(79) Sanpera, C.; Morera, M.; Ruiz, X.; Jover, L., Variability of Mercury and Selenium Levels in Clutches of Audouin's Gulls (Larus audouinii) Breeding at the Chafarinas Islands, Southwest Mediterranean. Arch Environ Contam Toxicol 2000, *39*, (1), 119-123.

(80) Bryan, A. L., Jr.; Hopkins, W. A.; Baionno, J. A.; Jackson, B. P., Maternal transfer of contaminants to eggs in common grackles (*Quiscalus quiscala*) nesting on coal fly ash basins. Arch. Environ. Contam. Toxicol. 2003, *45*, 273-277.

(81) Brasso, R. L.; Cristol, D. A., Effects of mercury exposure on the reproductive success of tree swallows (*Tachycineta bicolor*). Ecotoxicology 2008, *17*, (2), 133-141.

(82) Longcore, J. R.; Haines, T. A.; Halteman, W. A., Mercury in tree swallow food, eggs, bodies, and feathers at Acadia National Park, Maine, and an EPA Superfund site, Ayer, Massachusetts. Environ. Monit. Assess. 2007, *126*, (1-3), 129-143.

(83) Morrissey, C. A.; Eilliot, J. E.; Ormerod, S. J., Diet shifts during egg laying: implications for measuring contaminants in bird eggs. Environ. Pollut. 2010, *158*, 447-454.

(84) Nisbet, I. C. T., Eggshell characteristics and organochlorine residues in common terns: Variation with egg sequences. Colon Waterbirds 1982, *5*, 139-143.

(85) Bargellini, A.; Marchesi, I.; Rizzi, L.; Cauteruccio, L.; Masironi, R.; Simioli, M.; Borella, P., Selenium interactions with essential and toxic elements in egg yolk from commercial and fortified eggs. J. Trace Elem. Med. Biol. 2008, *22*, (3), 234-241.

(86) Zach, R., Hatching Asynchrony, Egg Size, Growth, and Fledging in Tree Swallows. The Auk 1982, *99*, (4), 695-700.

(87) Whittingham, L.; Dunn, P. O.; Lifjeld, J. T., Egg mass influences nestling quality in tree swallows, but there is no differential allocation in relation to laying order or sex. Condor 2007, *109*, 585-594.

TABLES AND FIGURES

TABLE 1.

F-statistics and P-values from one-tailed tests of the hypothesis that repeatability (R) was significantly greater than zero for all four trace elements in both species ($\alpha = 0.025$). Asterisks indicate whether the associated R-values (Fig. 3) were significantly greater than zero.

Element	F	df	p
Stinkpot			
Ba	61.8*	10	< 0.001
Se	39.4*	10	< 0.001
Sr	19.72*	10	< 0.001
Tl	318.57*	10	< 0.001
Tree Swallow			
Ba	2.16	11	0.031
Se	7.03*	11	< 0.001
Sr	9.42*	11	< 0.001
Tl	167.31*	11	< 0.001

FIGURE 1.

Mean trace element concentrations (mg/kg, dry mass) in stinkpot (white bars) and tree swallow (grey bars) egg contents from females co-located near a remediated coal fly ash spill in Kingston, TN, USA. Asterisks indicate significant differences between species.
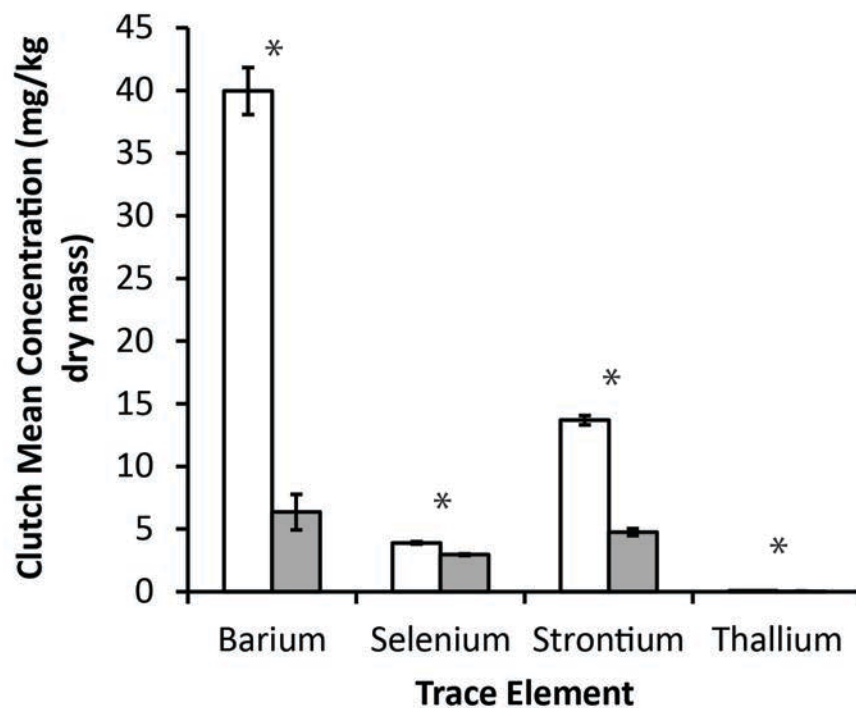
FIGURE 2.

Mean coefficients of variation (COV) of clutch trace element concentrations in stinkpot (white bars) and tree swallow (grey bars) eggs. Asterisks indicate significant differences between species.
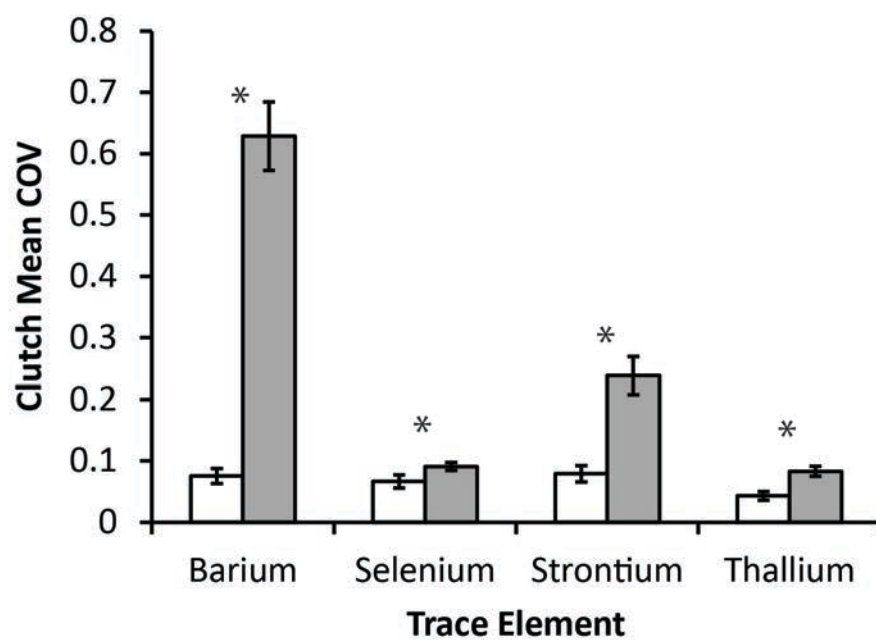
FIGURE 3.

Repeatabilities (R) of clutch trace element concentrations in stinkpot (white bars) and tree swallows (grey bars) eggs. Asterisks indicate significant differences between species.
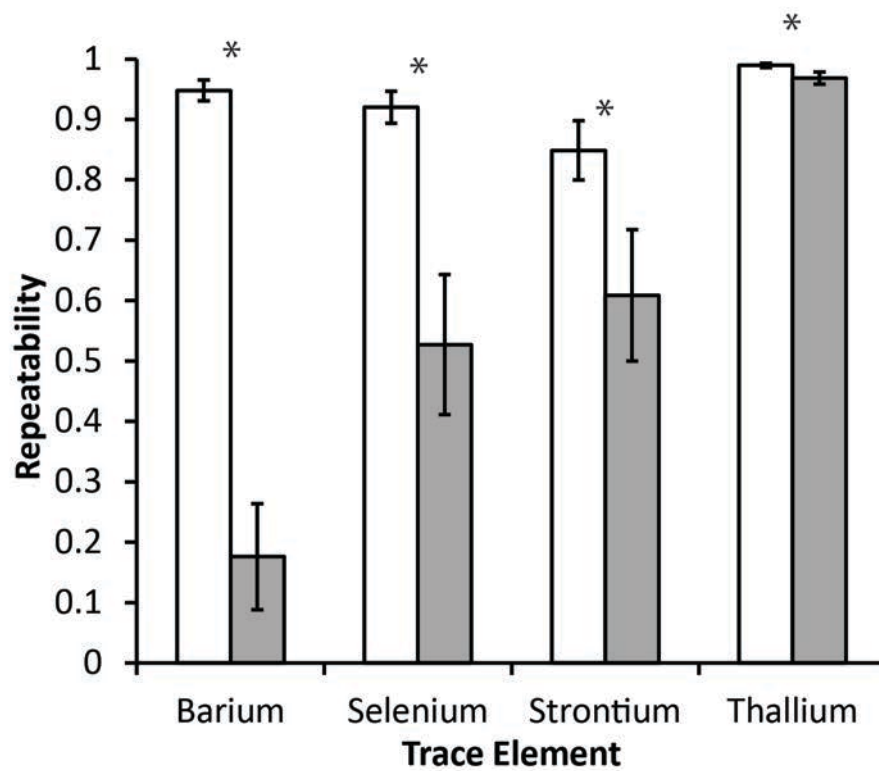
FIGURE 4.

Egg mean concentrations (white bars, left y-axes) and mean ranks of concentrations (gray bars, right y-axes) of Ba, Se, Sr, and Tl in tree swallow eggs. Eggs are ordered from first laid (1) to last laid (5). Letters indicate significant differences between eggs. Ranks were not statistically compared.

FIGURE 5.

Egg mean wet mass (A) and volume (B) in tree swallow eggs. Eggs are ordered from first laid (1) to last laid (5). Letters indicate significant differences between eggs.

CHAPTER 4 — INFLUENCE OF RELATIVE TROPHIC POSITION AND CARBON SOURCE ON SELENIUM BIOACCUMULATION IN TURTLES FROM A COAL FLY-ASH SPILL SITE
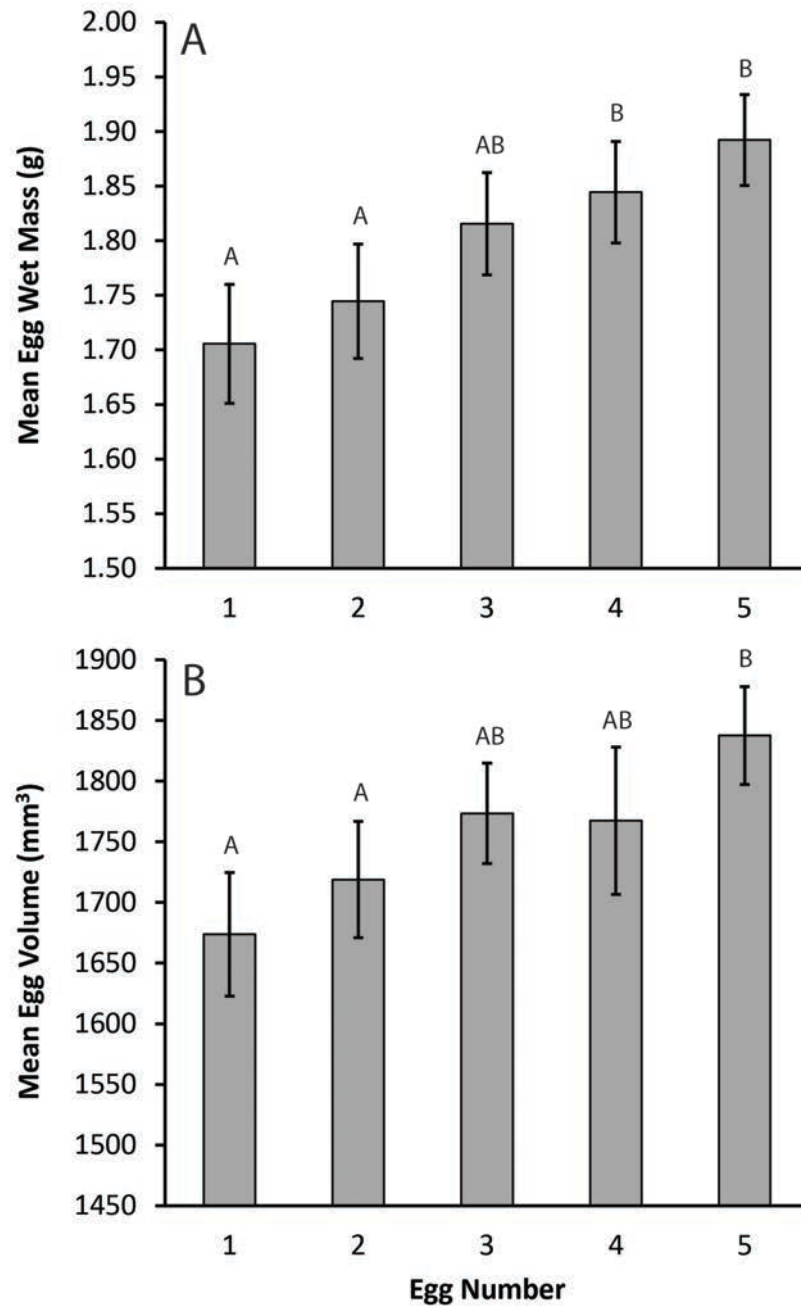
JAMES U. VAN DYKE

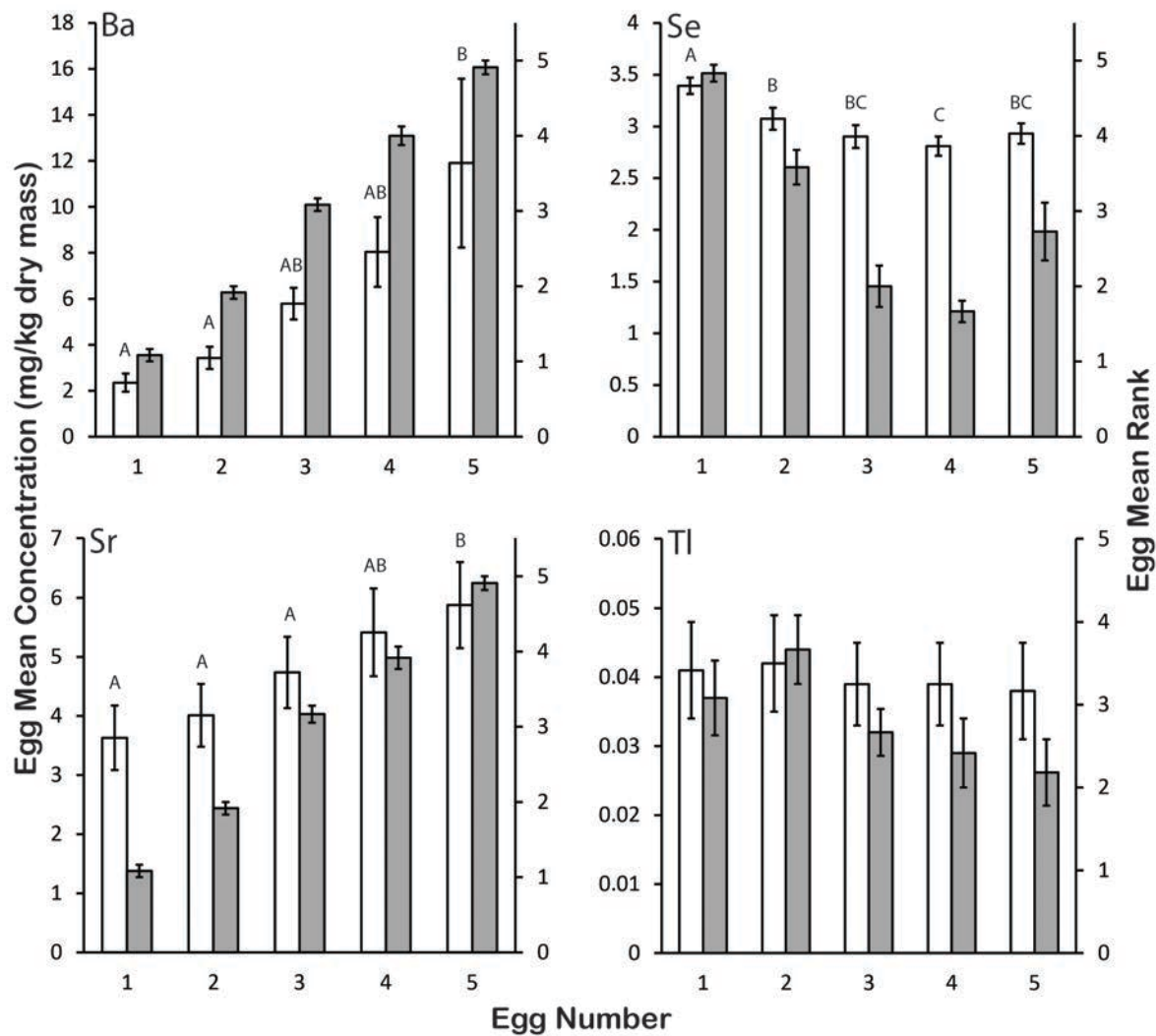100 Cheatham Hall, Department of Fish and Wildlife Conservation, Virginia Tech, Blacksburg, VA, 24061

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INTRODUCTION

Coal fly-ash contains elevated concentrations of many trace elements that can pose health risks to local plants, wildlife, and humans (Rowe et al., 2002). Selenium (Se) is a primary driver of ecological risk in aquatic systems impacted by coal fly-ash (Cherry and Guthrie, 1977, Hopkins et al., 2002, Rowe et al., 2002, Young et al., 2010). Unlike most other bioaccumulative pollutants, Se is an essential trace element in vertebrates at low concentrations, but becomes toxic at higher concentrations (Janz et al., 2010, Lemly, 1995, Tinggi, 2003). Toxicity may arise through multiple biochemical pathways (Janz et al., 2010), but most often manifests as reproductive impairment and/or teratogenicity (Janz et al., 2010, Ohlendorf et al., 1986).

Aquatic consumers are primarily exposed to ash-derived Se through assimilation from diet (Franz et al., 2011, Luoma et al., 1992, Skorupa and Ohlendorf, 1991). Primary producers biotransform inorganic Se (usually selenite) into selenomethionine in their tissues (Alaimo et al., 1994), which is biologically available to primary consumers and readily transferred throughout food webs (Jarman et al., 1996, Unrine et al., 2007a). However, although Se is known to bioaccumulate through dietary exposure, its propensity to biomagnify (i.e., increase concentration progressively with increasing trophic position; Dallinger et al., 1987) is less clear. The greatest enrichment of Se in aquatic food webs occurs during assimilation by primary producers (Presser and Luoma, 2010, Stewart et al., 2010). Enrichment of Se between subsequent trophic levels is dependent upon both its bioavailability in food and the assimilation efficiency of the consumer (Presser and Luoma, 2010). Thus, although Se concentrations are usually (but not always; Jardine and Kidd, 2011, Jarman et al., 1996) enriched between subsequent trophic levels (Ohlendorf et al., 1986, Presser and Luoma, 2010, Stewart et al., 2010), the magnitude of enrichment can differ among species within a given trophic level because of varying assimilation efficiency (Stewart et al., 2004). However, few studies of wild populations have traced among-species differences in Se bioaccumulation in higher consumers to among-species differences in primary consumers (Stewart et al., 2004).

Turtle species possess a suite of life history characteristics that make their assemblages useful for studying trophic influences on contaminant bioaccumulation (e.g., Bergeron et al., 2007, Congdon et al., 2008, Hopkins et al., 2013, Meyers-Schone and Walton, 1994). Turtles have small home ranges and long lifespans, and can be particularly susceptible to accumulating contaminants at impacted sites (Bergeron et al., 2007). Turtle species exhibit a diversity of dietary preferences (Ernst and Lovich, 2009), and are therefore likely to consume different prey types, with different body burdens of contaminants, within a polluted area (Hopkins, 2000). As ectotherms, turtles can subsist on relatively small amounts of prey, and can reach much greater population sizes than endotherms occupying similar trophic levels (Iverson, 1982), which makes them easy to sample.

In the current study, we examined the influence of feeding ecology on Se bioaccumulation in an assemblage of aquatic turtles inhabiting the Emory and Clinch River system in eastern Tennessee, USA. In December, 2008, 4.12 million cubic meters of coal fly-ash were accidentally discharged into the Emory-Clinch-Tennessee River system by the Tennessee Valley Authority's Kingston Fossil Plant (TVA, 2009). Subsequent remediation efforts removed the vast majority of ash prior to our study but ash-derived contaminants may still be entering local food webs. Following Bergeron et al., (2007) and Hopkins et al., (2013), we used stable isotopes (^{13}C and ^{15}N) from claws to test for among-species differences in relative carbon source and relative trophic positions. We then compared claw Se concentrations among species within the resulting trophic framework to determine whether relative carbon source and/or relative trophic position influenced bioaccumulation of ash-derived Se.

METHODS

SAMPLE COLLECTION

From April-July 2011, turtles were captured in the vicinity of the Kingston, TN Fossil Plant using hoop traps baited with sardines and/or chicken. All trapping occurred ~ 2.5 years after the spill event in December 2008 and ~ 1 year after the dredging efforts to remove ash from the river were completed in May, 2010. Traps were set in shallow-water areas (< 1 m deep) adjacent to microhabitats suitable for turtles. Trapping was concentrated in a contiguous 9.5 km length of river impacted by the fly-ash spill, including the Emory (river km 5.5-0.0) and Clinch Rivers (river km 7.0-3.0; Fig. 1). Traps were rebaited every 3 days, and were rotated among trapping locations depending upon trapping success. Captured turtles were removed from traps daily and transported to a field laboratory in Kingston, TN.

In the laboratory, turtle mass was measured with Pesola® scales, and carapace length, carapace width, and plastron length were measured using forestry calipers. The tips (top 2-3 mm) of all claws on the right rear foot (if present) were removed for analysis of Se, and the tips of all claws on the left rear foot (if present) were removed for stable isotope analysis. In several cases where turtles were missing all or a portion of a rear foot, the claw tips from the front feet were sampled instead. All turtles were released at the site of capture the day after processing. All claw samples were stored at -20° C. We used claws because they can be sampled non-invasively, grow continuously, and exhibit very long tissue turnover rates (~12 mo.; Aresco, 2005). Therefore, claw stable isotope composition and Se concentration should represent a temporal integration of both diet and Se bioaccumulation over the previous year (Bearhop et al., 2003, Hopkins et al., 2013, Hopkins et al., 2007).

TURTLE SPECIES

Claw stable isotope compositions and trace element concentrations were examined in seven species of turtles native to the Emory and Clinch Rivers near the site of the coal ash spill, including spiny softshell turtles (*Apalone spinifera*), snapping turtles (*Chelydra serpentina*), common map turtles (*Graptemys geographica*), Ouachita map turtles (*Graptemys ouachitensis*), common cooters (*Pseudemys concinna*), stinkpots (*Sternotherus odoratus*), and common sliders (*Trachemys scripta*). Although all of these freshwater turtles can be omnivorous to varying degrees, each differs in the relative proportions of prey types consumed (summarized from Ernst and Lovich, 2009). *Chelydra serpentina* can be highly piscivorous, and are likely to occupy the highest trophic position in most systems. *Apalone spinifera* are primarily carnivorous and are strongly sexually dimorphic in body size and diet; small males focus primarily on invertebrate prey, while large females are often more piscivorous. *Graptemys geographica* and *G. ouachitensis* are both medium-sized and feed primarily on mollusks. *Sternotherus odoratus* is a small omnivore that focuses on benthic invertebrates in soft-bottomed areas. Their benthic foraging strategy makes *S. odoratus* particularly susceptible to bioaccumulation of contaminants, especially mercury (Bergeron et al., 2007). *Trachemys scripta* are a medium-sized dietary generalist that opportunistically feed on both plants and animals. *Pseudemys concinna* are primarily herbivorous, and likely occupy the lowest trophic position within this assemblage.

STABLE ISOTOPE ANALYSIS

Stable isotope analysis followed the procedures established by Revesz and Qi (2006) and McCue and Pollock (2008). Claws sampled for stable isotope analysis were vortexed in millipore water to remove any external debris, and were dried to asymptotic mass at 50° C. Dried claws were stored with Drierite® dessicant until isotopic

analysis. At the University of Arkansas Stable Isotope Laboratory, 0.3-0.7 mg subsamples of claws were weighed on a Sartorius SC-2 nanobalance and wrapped in airtight 3 x 5 mm pressed tin capsules. If an entire set of claws weighed more than 0.7 mg, then claws were divided into subsamples which were individually analyzed and their values averaged together. Sealed sample-capsules were placed in a randomized order of analysis in 96-well microplates. All handling tools, surfaces, and weighing devices were cleaned with a methanol rinse and wiped with kimwipes after each sample. Standard reference materials (SRMs) were weighed and packaged in foil capsules in an identical manner, and were also added to the 96-well microplates. Six SRMs were added to the beginning of the microplate to precede all sample analyses. The first four SRMs were depleted in both ^{13}C and ^{15}N, while the latter two SRMs were enriched in both ^{13}C and ^{15}N. After the first 6 SRMs, claw samples were ordered in batches of nine, with every tenth sample being an additional SRM depleted in both ^{13}C and ^{15}N. An SRM depleted in both ^{13}C and ^{15}N was also added to the end of the microplate, and was the last sample analyzed on each microplate. A second microplate repeated the entire sequence. All microplates were sealed and stored in a dessicator until sample analysis.

Claw ^{15}N and ^{13}C contents were measured using a Finnigan Delta Plus continuous flow isotope ratio mass spectrometer (IR-MS) and elemental analyzer (EA). Samples sealed in tin cups were transferred from microplates and loaded in order into a microsampler on the EA. Under computer control, the autosampler dropped samples individually into a heated reaction tube in the EA, where they were combusted in a He atmosphere containing an excess of O_2 gas. Combustion converted total carbon and nitrogen from each sample into CO_2 and N_2 gas, respectively. From the EA, sample combustion gases were transported via He gas through a reaction furnace to remove excess O_2 gas and to convert any nitrous oxides into N_2, followed by a drying tube to remove water vapor. Carbon dioxide and N_2 gases were then separated by a gas chromatograph and introduced into the IR-MS through a Finnigan ConFlo II interface. The ConFlo II interface also introduces N_2 and CO_2 reference gases, and He gas for sample dilution. Mass and charge of the sample combustion gases measured by the IR-MS were uploaded to a PC and used to calculate $\delta^{13}C$ and $\delta^{15}N$ of each sample using Finnigan ISODAT 2.0 software. Isotopic determinations of $\delta^{13}C$ and $\delta^{15}N$ were normalized to a Vienna pee Dee belemnite standard and atmospheric N_2, respectively, using values provided by SRMs.

SELENIUM CONCENTRATION ANALYSIS

Claw Se concentrations were quantified using Inductively Coupled Mass Spectrometry (ICP-MS) at the Trace Element Analysis Core at Dartmouth College. Claws were stored at 4 ° C prior to sample preparation and analysis. Claws were first washed to remove external contamination. Individual claws were transferred to a 7ml polyethylene vial, 2 ml 1% solution of Triton X-100 was added and the vial was then placed in an ultrasonic bath for 20 minutes. The claw sample was washed 5 times with deionized water and then dried in the vial in a dry box. Each claw was then weighed into a pre-weighed VWR trace metal-clean polypropylene centrifuge tube and 0.5 ml of 9:1 HNO_3:HCl (Optima Grade, Fisher Scientific) was added. Individual claw weights were variable but were generally < 0.025 g. Claws were prepared for acid digestion in batches of 100 samples along with five each of blank, certified reference material, and fortified blank quality control samples. The very small sample masses prevented digestion and analysis of matrix duplicates and matrix duplicate spikes. All tubes were lightly capped and placed into a CEM MARS Express (Mathews, NC) microwave digestion unit for an open vessel digestion. A fiber optic temperature probe was placed into one of the sample tubes to provide temperature feedback to the MARS unit and the samples were heated to 95 °C with a ramp to temperature of 15 minutes and held at temperature for 45 minutes. The samples were then allowed to cool and 0.1 ml of H_2O_2 (Optima Grade, Fisher Scientific) was added and the samples were taken through

a further microwave heating program. The samples were then brought up to 10 ml with deionized water (Element QPod, Millipore, Billarica, MA). All measurements were recorded gravimetrically.

Digested samples were analyzed for Se by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Selenium (78) was measured in hydrogen mode (2.8 ml min^{-1}) and Se (82) was measured in He mode (4.8 ml min^{-1}) along with other analytes. Analytical procedures followed the general protocols outlined in EPA 6020A; the instrument was calibrated with NIST-traceable standards and calibration was verified with a second source traceable standard. The reporting limits were checked after calibration before the analysis of each batch of samples. The instrument reporting limit was 0.08 µg/L or 0.3 µg/L for Se 78 and Se 82 respectively corresponding to 0.095 mg/kg and 0.36 mg/kg dry mass average detection limits, respectively, for the claw samples. However, the detection limit for each claw is different and depends on the individual sample mass used in the digestion.   Sample QC included continuing calibration verification and blanks every 10 samples, analytical duplicates and analytical spikes.   Average recovery of certified reference material NIES Hair # 19, Se =1.79 mg/kg was 104 ± 7 % (n = 19), recovery of the fortified blank was 100 ± 11 % (n = 19), average recovery of the analysis spiked samples was 105 ± 7 % (n = 10) and relative percent deviation of the sample analysis duplicates was 6 ± 6 % (n = 10). The Se concentration of one *C. serpentina* sample was an order of magnitude greater than all other samples and was dropped from analysis because it was likely the result of a sampling error.

STATISTICAL ANALYSIS

Turtle claw $\delta^{13}C$ and $\delta^{15}N$ values were compared among species using a multivariate analysis of covariance (MANCOVA; PROC GLM, SAS 9.2). Sex was included in the model as a main effect, while river was included as a random effect. Due to low sample sizes and similar feeding habits (Ernst and Lovich, 2009), the two map turtle species (*Graptemys geographica* N = 7 and *G. ouachitensis* N = 8) were pooled together to improve statistical power. Turtle carapace length was included in the model as a covariate, because many turtles experience ontogenetic shifts in dietary preferences.

Among-species differences in claw Se concentration were examined using two approaches. First, ANCOVA was used to test for among-species differences in Se concentration, with carapace length, $\delta^{13}C$, and $\delta^{15}N$ as covariates. As in the isotope analysis, sex was included as a main effect, and river was included as a random effect. Both Se concentration and carapace length were log-transformed to improve normality. There were not enough degrees of freedom to test for significant differences in all 63 possible factors and interactions in a full factorial model, so we chose to examine the factorial interactions of all discrete factors (i.e., all interactive combinations of species, sex, and river), and all continuous factors (i.e., all interactive combinations of carapace length, $\delta^{13}C$, and $\delta^{15}N$). In addition, each possible bivariate interaction between discrete and continuous factors possible (e.g., species * carapace length, etc.) was examined to ensure that the assumption of slope homogeneity was met prior to ANCOVA. Second, ordinary least-squares regressions individually assessed the across-species relationships between $\delta^{13}C$ and Se concentration, and $\delta^{15}N$ and Se concentration. Selenium concentrations were log-transformed to improve normality in both regression models.

In all statistical tests, univariate normality and homoscedasticity of variance were assessed using normal probability plots and Shapiro-Wilk Tests. In all ANCOVAs, effects of the interactions between the covariate and main effects were examined before factor effects to ensure that the assumption of slope homogeneity was met. Sample sizes were not large enough to test for violations of multivariate normality, so Pillai's Trace was used as a test statistic

in all cases because it is the most robust to violations of multivariate normality (Scheiner, 2001). All statistical tests were judged at $\alpha = 0.05$, and all means are presented ± 1 SE.

RESULTS

TURTLES

During the 2011 trapping season, over 1000 turtles were captured, measured, and sampled. We compared claw Se concentrations, $\delta^{13}C$, and $\delta^{15}N$ from 20 *Apalone spinifera*, 20 *Chelydra serpentina*, 15 *Graptemys sp.* (7 *G. geographica* and 8 *G. ouachitensis*), 15 *Pseudemys concinna*, 19 *Sternotherus odoratus*, and 20 *Trachemys scripta*.

STABLE ISOTOPES

Turtle claw isotopic composition ($\delta^{13}C$ and $\delta^{15}N$) varied significantly with carapace length and with the interaction of river, sex, and species in the overall multivariate model (Table 1). To understand the causes of this complex interaction, the effects of each factor on $\delta^{13}C$ and $\delta^{15}N$ were examined in post-hoc univariate analyses. Mean $\delta^{13}C$ varied significantly with the interaction among river, sex, and species (Table 2) and was likely responsible for the significant interaction in the multivariate model. This significant interaction was driven primarily by male *P. concinna* from the Emory River (-19.07 $\pm$ 1.22 ‰), which were significantly more enriched in $\delta^{13}C$ relative to all other river-sex-species combinations (Tukey Test $p < 0.001$). The only other within-species differences observed were in *A. spinifera*, where mean $\delta^{13}C$ was significantly more enriched in males from the Clinch River (-27.44 $\pm$ 0.99 ‰) than in males from the Emory River (-30.10 $\pm$ 0.71 ‰; Tukey Test $p = 0.031$), and in *T. scripta*, where mean $\delta^{13}C$ was significantly more enriched in females from the Emory River (-24.35 $\pm$ 0.55 ‰) than in either males (-30.12 $\pm$ 1.74 ‰) or females (-27.73 $\pm$ 0.77 ‰) from the Clinch River (Tukey Test $p \leq 0.002$ in both cases). Overall, mean $\delta^{13}C$ varied significantly among species (Table 2; Fig. 2), and in the following fashion: *P. concinna* > *T. scripta* = *S. odoratus* = *C. serpentina* $\geq$ *A. spinifera* > *Graptemys*.

Mean $\delta^{15}N$ increased with carapace length in all species (Tables 1 and 2). Mean $\delta^{15}N$ also varied significantly among species (Table 2; Fig. 2), in the following fashion: *C. serpentina* = *Graptemys* sp. > *A. spinifera* = *T. scripta* = *S. odoratus* $\geq$ *P. concinna*. In addition to species differences, mean $\delta^{15}N$ was significantly higher in turtles from the Clinch River than in turtles from the Emory River (Table 2). However, mean $\delta^{15}N$ did not differ between males and females, and no interactions among factor effects were significant (Table 2).

SELENIUM CONCENTRATION

Turtle claw Se concentrations did not vary with any of the interactions examined (all two and three way interactions from ANCOVA $F \leq 3.72$ $p \geq 0.059$). Claw Se concentration varied significantly among species ($F_{5, 107} = 15.28$, $p < 0.001$), but did not vary with carapace length ($F_{1, 107} = 0.18$, $p = 0.669$), with $\delta^{13}C$ ($F_{1, 107} = 0.18$, $p = 0.676$), with $\delta^{15}N$ ($F_{1, 107} = 0.19$, $p = 0.667$), between rivers ($F_{1, 107} = 0.45$, $p = 0.503$), or between sexes ($F_{1, 107} = 1.49$, $p = 0.232$). Across species, mean claw Se concentrations were significantly higher in *A. spinifera* than in all other species (Fig. 3; Tukey Test $p < 0.001$), but did not differ among any other species examined (Fig. 3; Tukey Test $p \geq 0.444$). While ANCOVA did not reveal any significant relationships between claw Se concentration and claw $\delta^{13}C$ or $\delta^{15}N$ within species, ordinary least squares regression did reveal that Se concentrations significantly decreased with

increasing $\delta^{13}C$ across species (Fig. 4A; $F_{1, 107}$ = 31.14, p < 0.001). However, there was no significant relationship between $\delta^{15}N$ and claw Se concentration (Fig. 4B; $F_{1, 107}$ = 0.70, p = 0.406).

DISCUSSION

Claw Se concentrations significantly differed among species within our turtle assemblage. Because Se is assimilated primarily from diet (Luoma et al., 1992), Se concentration differences likely reflect differences in dietary preference. Our stable isotope results suggest that the species we studied significantly differ in their dietary preferences. However, Se concentrations differed only with relative carbon source, and not with relative trophic position. Here, we describe the trophic differences observed in our turtle assemblage, and then use the resulting trophic framework to explain among-species differences in Se concentration.

STABLE ISOTOPES

Stable isotope analyses generally supported the known dietary preferences of turtle species in our study. The ranges in individual $\delta^{15}N$ values (6.2 to 16.8 ‰) and mean species $\delta^{15}N$ values (8.3 to 13.2 ‰) suggested that turtles were feeding at more than one trophic level, assuming a 2-5 ‰ increment between successive trophic levels (Peterson and Fry, 1987, Post, 2002, Vander Zanden and Rasmussen, 2001). *Chelydra serpentina* and *Graptemys* species exhibited the most enriched $\delta^{15}N$, suggesting that they were feeding on the highest trophic level. *Chelydra serpentina* are primarily carnivorous and large individuals often eat fish, which occupy relatively high trophic levels themselves (Ernst and Lovich, 2009), so their placement at the highest trophic level is not surprising. *Graptemys* are highly carnivorous as well but typically feed on bivalves (Ernst and Lovich, 2009). *Pseudemys concinna* exhibited the most depleted $\delta^{15}N$ values (9.4 ‰), which suggests that they were feeding on the lowest relative trophic position. *Pseudemys concinna* is primarily herbivorous (Ernst and Lovich, 2009), so its placement at the lowest trophic level is consistent with its known feeding ecology.

Relative to the extremes mentioned above, *A. spinifera*, *S. odoratus*, and *T. scripta* exhibited intermediate and overlapping $\delta^{15}N$ values. *Apalone spinifera* and *S. odoratus* feed primarily on invertebrates (Ernst and Lovich, 2009), and would be expected to overlap with each other. However, it is surprising that their mean $\delta^{15}N$ values (10.9 ‰ and 10.6 ‰, respectively) were significantly depleted relative to those of *Graptemys*, which also primarily feed on invertebrates. The discrepancy could reflect differences in dietary specialization; *Graptemys* often specialize on bivalves, while *A. spinifera* and *S. odoratus* often feed more broadly on a diversity of invertebrates (Ernst and Lovich, 2009). *Trachemys scripta* is likely the most generalist species in our study, and its intermediate overall mean $\delta^{15}N$ (10.9 ‰) could be due to its propensity to feed opportunistically on both plant and animal matter.

In addition to the observed species differences, $\delta^{15}N$ was significantly higher in all turtles from Clinch River than from the Emory River, regardless of species. This result is somewhat surprising because our study site comprises a fairly small area (Fig. 1), and preliminary mark-recapture data suggest that individuals of some species are capable of moving between rivers (Steen et al., unpublished data). Given the long time required for claw tissue turnover in turtles (~12 mo.; Aresco, 2005), our data suggest that even if individual turtles travel between the Emory and Clinch Rivers, the majority of their feeding may be restricted to only one river. Ultimately, the between-river difference in $\delta^{15}N$ suggests either that turtles feed at slightly different trophic positions in the Emory and Clinch Rivers, or that relative baseline Nitrogen isotopic enrichment differs between the two rivers. The latter seems more likely

because the Clinch River drains a much larger watershed than the Emory River (including the Emory itself) and has a greater potential for anthropogenic disturbance (Atchley et al., 2000, Burr et al., 2000), both of which may increase $\delta^{15}N$ in aquatic primary producers (Cabana and Rasmussen, 1996, March and Pringle, 2003).

As with $\delta^{15}N$, we also found differences in $\delta^{13}C$, both among species and between rivers. ^{13}Carbon does not fractionate with successive trophic levels, but differs with producer photosynthetic pathway (C_3, C_4), and has been used to differentiate the ultimate sources of carbon among species (Peterson and Fry, 1987, Post, 2002). Individual $\delta^{13}C$ values ranged from -32.5 to -17.5 ‰, and species means ranged from -30.7 to -23.5 ‰. Overall, our results suggested that *P. concinna* were feeding on more enriched carbon sources than all other species, while *Graptemys* were feeding on more depleted carbon sources, and *A. spinifera*, *C. serpentina*, *S. odoratus*, and *T. scripta* were feeding on carbon sources that were intermediate in carbon enrichment. These differences likely reflect different rates of consumption of C_3 and C_4 producers at the bases of food chains utilized by different species (Peterson and Fry, 1987, Post, 2002).

SELENIUM

Although our $\delta^{15}N$ results suggest that turtles in the Emory-Clinch River system are feeding at different trophic levels, we observed no relationship between relative trophic position and Se concentration. Selenium concentrations did not change consistently with relative trophic position; therefore we found no evidence of biomagnification of Se in this turtle assemblage. Indeed, the species at the highest trophic levels, *C. serpentina* and *Graptemys*, exhibited Se concentrations similar to the species at the lowest trophic level, *P. concinna. Apalone spinifera*, which fed at an intermediate trophic level, exhibited significantly higher Se concentrations than all other species. Furthermore, *S. odoratus* and *T. scripta* fed at intermediate trophic levels similar to that of *A. spinifera*, but did not exhibit elevated Se concentrations. Thus, our findings suggest that *Apalone spinifera* consume different food items at that trophic level, which are relatively enriched in Se. Similarly, Orr et al., (2006) found that invertebrate species feeding at identical trophic positions exhibited highly variable Se concentrations in an impacted system. Notably, there are a number of invertebrate and vertebrate primary and secondary consumers present in the Tennessee River system, including bivalves, gastropods, insects, fish, and larval amphibians. Among-taxa differences in Se enrichment within this assemblage could explain our observation. Alternatively, if Se concentrations are not different among turtle prey at a given trophic level, our results might suggest that Se assimilation efficiencies differ among turtle species, as has been observed in some invertebrates (Stewart et al., 2004). Another possibility is that turtles exhibiting enriched $\delta^{13}C$ may be relying more heavily on terrestrial carbon sources (Willson et al., 2010), which may be low in Se concentrations if the primary producers are above the water line and thus outside of areas contaminated by the fly-ash spill. In addition, prior studies have reported that mercury and selenium antagonistically affect each other's bioaccumulation (Khan and Wang, 2009, Reash, 2012), but preliminary analyses revealed no such effect in this system (Van Dyke et al., *unpublished*). Our work corroborates prior studies on other taxonomic groups that reported a lack of evidence for Se biomagnification among consumers at different relative trophic levels (Jardine and Kidd, 2011, Jarman et al., 1996, Ohlendorf et al., 1986, Orr et al., 2012, Unrine et al., 2007b).

Selenium concentrations decreased with increasing $\delta^{13}C$ across all turtle species, suggesting that Se bioaccumulation differed with relative carbon source. Importantly, the relationship remained significant even if *A. spinifera* were removed from the analysis (P < 0.001). Jardine and Kidd (2011) suggested that Se concentrations might change with $\delta^{13}C$ if $\delta^{13}C$ were considered a proxy for distance from a Se contamination site. In our system, $\delta^{13}C$ differed between rivers only within *A. spinifera*, *S. odoratus*, and *T. scripta*, but we found no evidence for within-

species relationships between δ^{13}C and Se concentration. Therefore, it seems unlikely that the Se differences we observed were due to site differences in Se contamination in any species in our study. Alternatively, turtle Se concentrations might be influenced by variable Se assimilation or retention rates in different primary producers. All aquatic producers are thought to assimilate Se with relative ease (Ornes et al., 1991, Stewart et al., 2010), but the differences in turtle Se concentrations and relative carbon source could indicate that aquatic producers that are depleted in ^{13}C might concentrate Se at a greater rate than do producers that are enriched in ^{13}C. Littoral periphython and aquatic plants are typically enriched in ^{13}C relative to benthic algae and phytoplankton (France, 1995a, 1995b). Therefore those turtles exhibiting high Se concentrations and depleted ^{13}C may be feeding predominately from food webs originating from benthic algae and phytoplankton, while those exhibiting low Se concentrations and enriched ^{13}C may be feeding predominately from food webs originating from periphython and aquatic plants. However, variable Se trophic transfer rates between different species of primary producers and primary consumers likely also play a role in the observed pattern (Stewart et al., 2010).

Although mean Se concentrations in *A. spinifera* were higher than those of other species, the relationship between claw Se concentration and health effects have not been determined in any vertebrate. Thus, the toxicological significance of the Se concentrations reported here is unknown. Forthcoming examinations of turtle reproductive success will determine whether the turtles in this system face any adverse consequences of Se bioaccumulation. In addition, forthcoming examinations of temporal effects on Se concentrations will determine whether rapid remediation efforts successfully averted further Se bioaccumulation in turtles.

CONCLUSIONS

Our results demonstrate that relative trophic position does not predict Se bioaccumulation in an assemblage of closely-related vertebrates exposed to a recently remediated coal fly-ash spill. Our results contribute to a growing body of evidence that Se does not consistently biomagnify in higher-order consumers (Jardine and Kidd, 2011, Jarman et al., 1996, Ohlendorf et al., 1986, Orr et al., 2012). However, Se concentrations did decrease with increasing δ^{13}C across all species. Delta-^{13}C is determined by ^{13}C fractionation rates during photosynthesis in primary producers at the base of the food web (Peterson and Fry, 1987, Post, 2002). Therefore, our results suggest that Se may be more bioavailable to turtles feeding on food webs in which aquatic producers are depleted in δ^{13}C (i.e., benthic algae and phytoplankton) than to those feeding on food webs in which producers are enriched in δ^{13}C (i.e., littoral periphyton and aquatic plants). Although Se bioaccumulation rate is known to vary among primary producers (Stewart et al., 2010), prior studies of trophic enrichment have focused on food webs based on a single primary producer species (Besser et al., 1993, Conley et al., 2009, Orr et al., 2012, Stewart et al., 2004), or have not been able to differentiate species differences from abiotic effects associated with differences in preferred habitat (Orr et al., 2006). Thus, the possibility that variability in Se bioaccumulation among syntopic producer species might contribute to differences in Se exposure at higher trophic levels has been relatively unexplored. Elucidating these relationships is critical to advancing understanding of Se trophic dynamics.

REFERENCES

Alaimo, J., Ogle, R.S., Knight, A.W., 1994. Selenium uptake by larval *Chironomus decorus* from a *Ruppia maritima*-Based benthic/detrital substrate. Archives of Environmental Contamination and Toxicology 27, 441-448.

Aresco, M.J., 2005. Ecological relationships of turtles in northern Florida lakes: a study of omnivory and the structure of a lake food web. Doctoral Dissertation Thesis, Florida State University, Tallahassee, FL, 141 pp.

Atchley, M., Burr, J., Harris, N., Jones, K., Kamel, F., Price, J., Sanaat, H., Stodola, P., West, J., Williams, A., Cochran, R.E., Duhl, D., Fathi, S., Vann, A., Wang, S., 2000. Emory River watershed (06010208) of the Tennessee River Basin. In: Tennessee Department of Environment and Conservation, D.o.W.P.C., Watershed Management Section (Ed.), Water Quality Management Plan.

Bearhop, S., Furness, R.W., Hilton, G.M., Votier, S.C., Waldron, S., 2003. A forensic approach to understanding diet and habitat use from stable isotope analysis of (avian) claw material. Functional Ecology 17, 270-275.

Bergeron, C.M., Husak, J.F., Unrine, J.M., Romanek, C.S., Hopkins, W.A., 2007. Influence of feeding ecology on blood mercury concentrations in four species of turtles. Environmental Toxicology and Chemistry 26 (8), 1733-1741.

Besser, J.M., Canfield, T.J., La Point, T.W., 1993. Bioaccumulation of organic and inorganic selenium in a laboratory food chain. Environmental Toxicology and Chemistry 12, 57-72.

Burr, J., Atchley, M., Everett, L., Turaski, S., Cochran, R.E., Duhl, D., McGahen, R., Wang, S., 2000. Lower Clinch River watershed (06010207) of the Tennessee River Basin. In: Tennessee Department of Environment and Conservation, D.o.W.P.C., Watershed Management Section (Ed.), Water Quality Management Plan.

Cabana, G., Rasmussen, J.B., 1996. Comparison of aquatic food chains using nitrogen isotopes. Proceedings of the National Academy of Sciences of the United States of America 93, 10844-10847.

Cherry, D.S., Guthrie, R.K., 1977. Toxic metals in surface waters from coal ash. Water Resources Bulletin 13, 1227-1236.

Congdon, J.D., Greene, J.L., Brooks, R.J., 2008. Reproductive and nesting ecology of female snapping turtles. In: Steyermark, A.C., Finkler, M.S., Brooks, R.J. (Eds.), Biology of the Snapping Turtle. Johns Hopkins University Press, Baltimore, MD, pp. 123-134.

Conley, J.M., Funk, D.H., Buchwalter, D.B., 2009. Selenium bioaccumulation and maternal transfer in the mayfly *Centroptilum triangulifer* in a life-cycle, periphyton-biofilm trophic assay. Environmental Science & Technology 43, 7952-7957.

Dallinger, R., Prosi, F., Segner, H., Back, H., 1987. Contaminated food and uptake of heavy metals by fish: a review and a proposal for further research. Oecologia (Berlin) 73 (1), 91-98.

Ernst, C.H., Lovich, J.E., 2009. Turtles of the United States and Canada. Johns Hopkins University Press, Baltimore, MD.

France, R.L., 1995a. Carbon-13 enrichment in benthic compare to planktonic algae: foodweb implications. Marine Ecology Progress Series 124, 307-312.

France, R.L., 1995b. Differentiation between littoral and pelagic food webs in lakes using stable carbon isotopes. Limnology and Oceanography 40 (7), 1310-1313.

Franz, E.D., Wiramanaden, C.I.E., Janz, D.M., Pickering, I.J., Liber, K., 2011. Selenium bioaccumulation and speciation in *Chironomus dilutus* exposed to water-borne selenate, selenite, or seleno-DL-methionine. Environmental Toxicology and Chemistry 30 (10), 2292-2299.

Hopkins, W.A., 2000. Reptile toxicology: opportunities and challenges on the last frontier of vertebrate ecotoxicology. Environmental Toxicology and Chemistry 19 (10), 2391-2393.

Hopkins, W.A., Bodinof, C., Budischak, S., Perkins, C., 2013. Nondestructive indices of mercury exposure in three species of turtles occupying different trophic niches downstream from a former chloralkali facility. Ecotoxicology 22 (1), 22-32.

Hopkins, W.A., Hopkins, L.B., Unrine, J.M., Snodgrass, J.W., Elliot, J.D., 2007. Mercury concentrations in tissues of osprey from the Carolinas, USA. Journal of Wildlife Management 71 (6), 1819-1829.

Hopkins, W.A., Snodgrass, J.W., Roe, J.H., Staub, B.P., Jackson, B.P., Congdon, J.D., 2002. Effects of food ration on survival and sublethal responses of lake chubsuckers (*Erimyzon sucetta*) exposed to coal combustion wastes. Aquatic Toxicology (Amsterdam) 57, 191-202.

Iverson, J.B., 1982. Biomass in turtle populations - a neglected subject. Oecologia (Berlin) 55, 69-76.

Janz, D.M., DeForest, D.K., Brooks, M.L., Chapman, P.M., Gilron, G., Hoff, D., Hopkins, W.A., McIntyre, D.O., Mebane, C.A., Palace, V.P., Skorupa, J.P., Wayland, M., 2010. Selenium toxicity to aquatic organisms. In: Chapman, P.M., Adams, W.J., Brooks, M.L., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P. (Eds.), Ecological Assessment of Selenium in the Aquatic Environment. CRC Press, Boca Raton, FL, USA, pp. 141-231.

Jardine, T.D., Kidd, K.A., 2011. Low concentrations of selenium in stream food webs of eastern Canada. Science of the Total Environment 409, 785-791.

Jarman, W.M., Hobson, K.A., Sydeman, W.J., Bacon, C.E., McLaren, E.B., 1996. Influence of trophic position and feeding location on contaminant levels of the Gulf of Farallones food web revealed by stable isotope analysis. Environmental Science & Technology 30, 654-660.

Khan, M.A.K., Wang, F., 2009. Mercury-selenium compounds and their toxicological significance: toward a molecular understanding of the mercury-selenium antagonism. Environmental Toxicology and Chemistry 28 (8), 1567-1577.

Lemly, A.D., 1995. A protocol for aquatic hazard assessment of selenium. Ecotoxicology and Environmental Safety 32, 280-288.

Luoma, S.N., Johns, C., Fisher, N.S., Steinberg, N.A., Oremland, R.S., Reinfelder, J.R., 1992. Deterimination of selenium bioavailability to a benthic bivalve from particulate and solute pathways. Environmental Science & Technology 26, 485-491.

March, J.G., Pringle, C.M., 2003. Food web structure and basal resource utilization along a tropical island stream continuum, Puerto Rico. Biotropica 35 (1), 84-93.

McCue, M.D., Pollock, E.D., 2008. Stable isotopes may provide evidence for starvation in reptiles. Rapid Communications in Mass Spectrometry 22, 2307-2314.

Meyers-Schone, L., Walton, B.T., 1994. Turtles as monitors of chemical contaminants in the environment. Reviews of Environmental Contamination and Toxicology 135, 93-153.

Ohlendorf, H.M., Hoffman, D.J., Saiki, M.K., Aldrich, T.W., 1986. Embryonic mortality and abnormalities of aquatic birds: apparent impacts of selenium from irrigation drainwater. Science of the Total Environment 52, 49-63.

Ornes, W.H., Sajwan, K.S., Dosskey, M.G., Adriano, D.C., 1991. Bioaccumulation of selenium by floating aquatic plants. Water Air and Soil Pollution 57&58, 53-57.

Orr, P.L., Guiguer, K.R., Russel, C.K., 2006. Food chain transfer of selenium in lentic and lotic habitats of a western Canadian watershed. Ecotoxicology and Environmental Safety 63, 175-188.

Orr, P.L., Wirmanaden, C.I.E., Paine, M.D., Franklin, W., Fraser, C., 2012. Food chain model based on field data to predict westslope cutthroat trout (*Oncorhynchus clarkii*) ovary selenium concentrations from water selenium concentrations in the Elk Valley, British Columbia. Environmental Toxicology and Chemistry 31 (3), 672-680.

Peterson, B.J., Fry, B., 1987. Stable isotopes in ecosystem studies. Annual Review of Ecology and Systematics 18, 293-320.

Post, D.M., 2002. Using stable isotopes to estimate trophic position: models, methods, and assumptions. Ecology 83 (3), 703-718.

Presser, T.S., Luoma, S.N., 2010. A methodology for ecosystem-scale modeling of selenium. Integrated Environmental Assessment and Management 6 (4), 685-710.

Reash, R.J., 2012. Selenium, arsenic, and mercury in fish inhabiting a fly ash exposure gradient: interspecific bioaccumulation patterns and elemental associations. Environmental Toxicology and Chemistry 31 (4), 739-747.

Revesz, K., Qi, H., 2006. Determination of the $\delta(^{15}N/^{14}N)$ and $\delta(^{13}C/^{12}C)$ of total N and C in Solids: RSIL Lab Code 1832. In: Revesz, K., Coplen, T.B. (Eds.), Methods of the Reston Stable Isotope Laboratory. Techniques and Methods. U.S. Geological Survey, Reston, VA.

Rowe, C.L., Hopkins, W.A., Congdon, J.D., 2002. Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: A review. Environmental Monitoring and Assessment 80, 207-276.

Scheiner, S.M., 2001. MANOVA: Multiple response variables and multispecies interactions. In: Scheiner, S.M., Gurevitch, J. (Eds.), Design and Analysis of Ecological Experiments. Oxford University Press, New York, NY, pp. 99-115.

Skorupa, J.P., Ohlendorf, H.M., 1991. Contaminants in drainage water and avian risk thresholds. In: Dinar, A., Ziberman, D. (Eds.), The economics and management of water and drainage in agriculture. Kluwer Academic Publishers, Boston, MA, pp. 345-368.

Stewart, A.R., Luoma, S.N., Schlekat, C.E., Doblin, M.A., Hieb, K.A., 2004. Food web pathway determines how selenium affects aquatic ecosystems: a San Francisco Bay case study. Environmental Science & Technology 38, 4519-4526.

Stewart, A.R., Grosell, M., Buchwalter, D., Fisher, N., Luoma, S., Mathews, T., Orr, P., Wang, W., 2010. Bioaccumulation and trophic transfer of selenium. In: Chapman, P.M., Adams, W.J., Brooks, M.L., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P. (Eds.), Ecological Assessment of Selenium in the Aquatic Environment. CRC Press, Boca Raton, FL, USA, pp. 93-139.

Tinggi, U., 2003. Essentiality and toxicity of selenium and its status in Australia: a review. Toxicology Letters (Shannon) 137, 103-110.

TVA, 2009. Corrective Action Plan for the TVA Kingston Fossil PLant Ash Release, Kingston, TN.

Unrine, J.M., Jackson, B.P., Hopkins, W.A., 2007a. Selenomethionine biotransformation and incorporation into proteins along a simulated terrestrial food chain. Environmental Science & Technology 41, 3601-3606.

Unrine, J.M., Hopkins, W.A., Romanek, C.S., Jackson, B.P., 2007b. Bioaccumulation of trace elements in omnivorous amphibian larvae: implications for amphibian health and contaminant transport. Environmental Pollution 149, 182-192.

Vander Zanden, M.J., Rasmussen, J.B., 2001. Variation in $\delta^{15}N$ and $\delta^{13}C$ trophic fractionation: implications for aquatic food web studies. Limnology and Oceanography 46 (8), 2061-2066.

Willson, J.D., Winne, C.T., Pilgrim, M.A., Romanek, C.S., Gibbons, J.W., 2010. Seasonal variation in terrestrial resource subsidies influences trophic niche width and overlap in two aquatic snake species: a stable isotope approach. Oikos 119, 1161-1171.

Young, T.F., Finley, K., Adams, W.J., Besser, J., Hopkins, W.A., Jolley, D., McNaughton, E., Presser, T.S., Shaw, D.P., Unrine, J., 2010. What you need to know about Selenium. In: Chapman, P.M., Adams, W.J., Brooks, M.L., Delos, C.G., Luoma, S.N., Maher, W.A., Ohlendorf, H.M., Presser, T.S., Shaw, D.P. (Eds.), Ecological Assessment of Selenium in the Aquatic Environment. CRC Press, Boca Raton, FL, USA, pp. 7-4

TABLES AND FIGURES

TABLE 1.

Results of MANCOVA analysis of mean claw $\delta^{13}C$ and $\delta^{15}N$ sampled from turtles from the vicinity of the coal fly-ash spill in the Emory and Clinch Rivers, Tennessee. The interaction between carapace length, river, sex, and species tests the assumption of homogeneity of slope necessary to ANCOVA, and no other interactions with carapace length were examined. Error degrees of freedom was 114 in all cases, and asterisks indicate factors significant at $\alpha = 0.05$.

Source	Pillai's Trace	F	df	p
Carapace Length*	0.161	7.49	2	0.001
River*	0.193	9.32	2	< 0.001
Sex	0.037	1.52	2	0.226
Species*	0.738	9.23	10	< 0.001
River X Sex	0.042	1.71	2	0.187
River X Species*	0.347	3.31	10	< 0.001
Sex X Species	0.175	1.52	10	0.137
River X Sex X Species*	0.233	2.08	10	0.029
Carapace Length X River X Sex X Species	0.077	0.57	8	0.800

TABLE 2.

Results of post-MANCOVA univariate analyses of claw $\delta^{13}C$ and $\delta^{15}N$ sampled from turtles from the vicinity of the coal fly-ash spill in the Emory and Clinch Rivers, Tennessee. Error degrees of freedom was 103 in all cases, and asterisks indicate factors significant at $\alpha = 0.05$.

Source	F	df	p
$\delta^{13}C$			
Carapace Length	1.83	1	0.180
River*	8.17	1	0.005
Sex	0.88	1	0.351
Species*	19.39	5	< 0.001
River X Sex	2.94	1	0.090
River X Species*	5.31	5	< 0.001
Sex X Species*	2.66	5	0.028
River X Sex X Species*	3.85	5	0.004
$\delta^{15}C$			
Carapace Length*	12.14	1	< 0.001
River*	12.70	1	< 0.001
Sex	2.47	1	0.120
Species*	7.62	5	< 0.001
River X Sex	0.83	1	0.366
River X Species	2.07	5	0.078
Sex X Species	0.42	5	0.835
River X Sex X Species	1.29	5	0.759

FIGURE 1.

Map of the turtle sampling area near Kingston, TN. Note that the Emory River is a tributary of the Clinch River, which is itself a tributary of the Tennessee River. Markers (+) and associated numbers indicate river kilometers, which are reported as distances from the downstream terminus of each river. The coal fly-ash spill of 22 December 2008 occurred at approximately Emory River kilometer 4, in and around the Swan Pond Embayment, which flows into the Emory River proper. All of the sampling reported in this study occurred from Emory River kilometers 5.5-0, and Clinch River kilometers 7-3 and represents a contiguous length of 9.5 river kilometers, approximately 8 of which are downstream of the spill site.
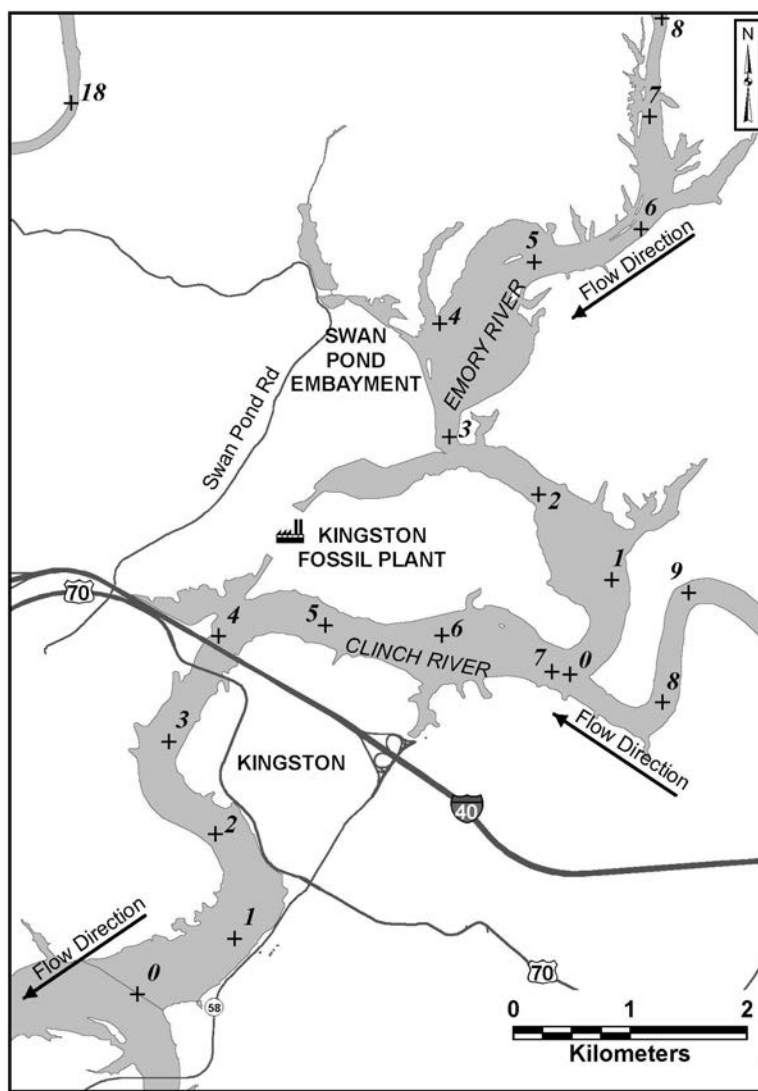
FIGURE 2.

Isotopic compositions (δ^{13}C and δ^{15}N) of turtle claws sampled from turtle species inhabiting Emory and Clinch Rivers near the site of the Kingston coal fly-ash spill. Both δ^{13}C and δ^{15}N varied significantly among species. Symbols represent least-squares means for each species corrected for carapace length. Significant differences among rivers and/or sexes are not shown for simplicity. Letters indicate significant differences among species in δ^{13}C (Tukey Test $p \leq 0.015$ for significant differences, $p \geq 0.074$ for non-significant differences), while numbers indicate among-species significant differences in δ^{15}N (Tukey Test $p \leq 0.023$ for significant differences, $p \geq 0.063$ for non-significant differences). Error bars represent ± 1 SE.
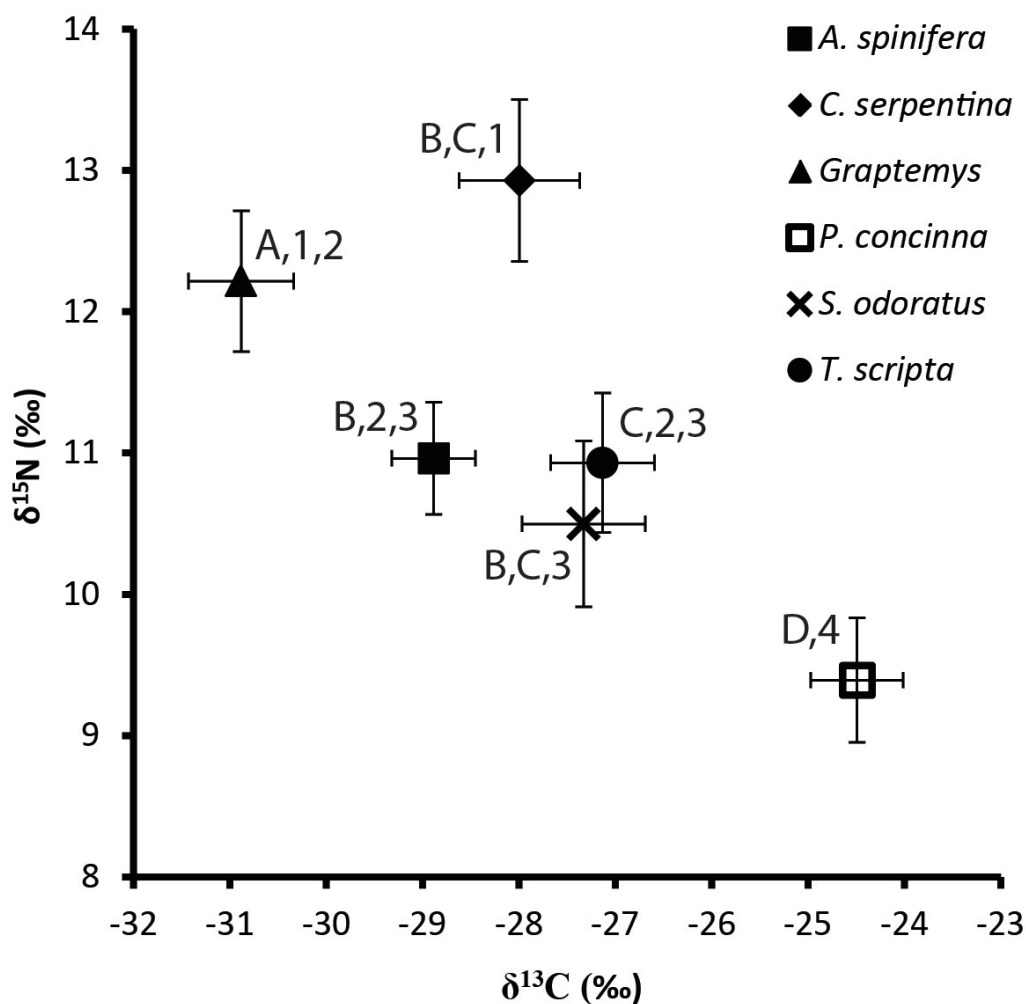
FIGURE 3.

Claw Se concentrations sampled from turtle species inhabiting Emory and Clinch Rivers near the site of the Kingston coal fly-ash spill. Mean Se concentrations of turtle claws were significantly higher in *Apalone spinifera* than in all other species. Error bars represent ± 1 SE and the asterisk represents a significant difference.
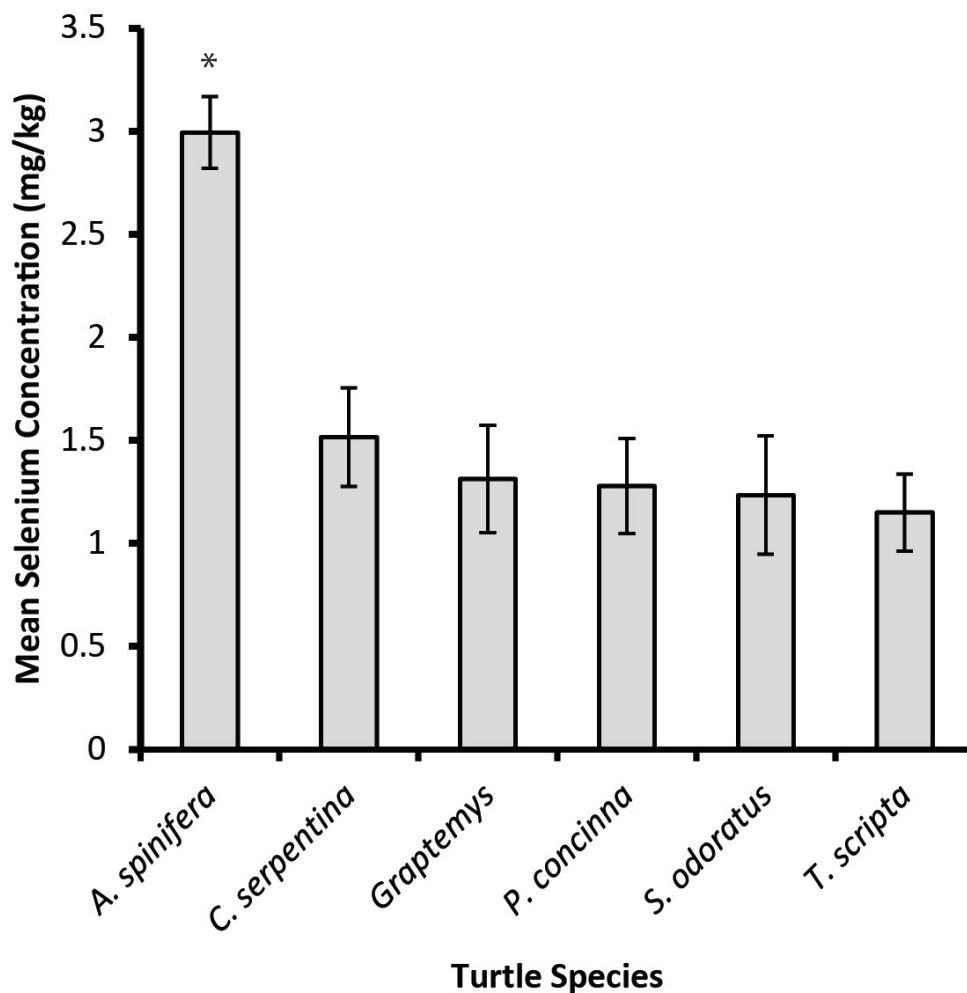
FIGURE 4.

Log-transformed Se concentrations are shown regressed against δ^{13}C (A) and δ^{15}N (B). Small symbols represent values for individual turtles, while larger symbols with error bars represent species means ± 1 SE. The relationship between log-transformed Se concentration and **log-transformed δ^{13}C** was significant, and is best explained by the equation: $LOG(Se) = -0.038(\delta^{13}C) - 0.906$ ($F_{1, 107} = 31.14$ $p < 0.001$; $r^2 = 0.21$). There was no significant relationship between log-transformed Se concentration and log-transformed δ^{15}N ($F_{1, 107} = 0.23$ $p = 0.602$).
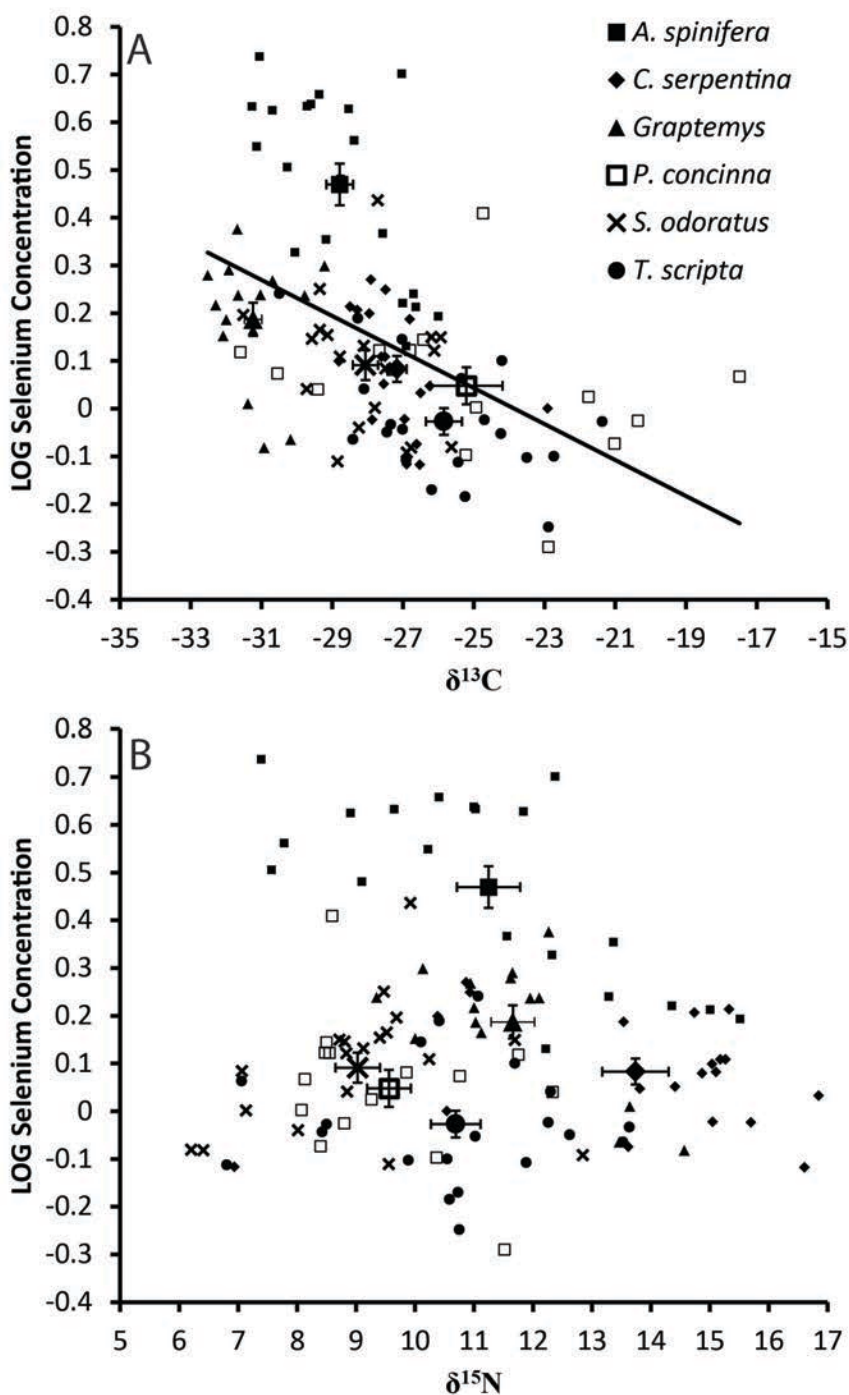
CHAPTER 5
FRESHWATER TURTLE ABUNDANCE, MORPHOLOGY AND SEX RATIO IN RELATION TO A REMEDIATED COAL FLY-ASH SPILL

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INTRODUCTION

In December 2008, 4.1 million cubic meters of coal fly ash were accidentally discharged into the Emory River in the vicinity of Kingston, TN (TVA 2009). Ash particles from the spill were swept downstream to the Clinch River and eventually were detected in the Tennessee River over 12.9 km downstream of the spill. Because coal ash contains elevated concentrations of many trace elements, including arsenic (As), cadmium (Cd), selenium (Se), strontium (Sr), and vanadium (V), the spill may pose health risks to local plants, wildlife, and humans (Rowe et al. 2002). In the time since the spill, the majority of coal ash has been removed from the Emory-Clinch-Tennessee River system through massive remediation efforts, but residual ash remains in some locations. Thus, the potential for coal ash constituents to enter local food webs and affect local biota remains a concern. Monitoring over temporal and spatial scales is necessary to determine the locations at which wildlife face risks of exposure to residual ash, and integrative studies of contaminant effects are necessary to determine the ecological impacts of the coal ash spill (e.g., Hopkins and Rowe 2010).

Turtles are excellent model organisms for assessing the effects of ash-derived trace element exposure in vertebrates. Turtle species can be herbivorous, carnivorous, and/or omnivorous at different life stages (Ernst and Lovich 2009), and may bioaccumulate trace elements through their diet. Turtle species that occupy high trophic levels may be especially at risk of toxicant biomagnification, because some contaminants can increase in tissue concentration with increases in trophic position (Meyers- Schone and Walton 1994, Bergeron et al. 2007). Many turtle species are long-lived (Congdon et al. 2008, Ernst and Lovich 2009), and as a result may be at significant risk of long-term exposure to trace elements. Compared to many other vertebrates, turtles are relatively sedentary and have small home ranges, which make them excellent model species for determining spatial distributions of contaminant effects (Hopkins 2000). As ectotherms, turtles can subsist on relatively small amounts of prey and can reach greater population sizes than endotherms occupying similar trophic levels (Iverson 1982). In addition, some turtles are highly fecund, with some species of turtles laying large numbers of eggs multiple times per year (Ernst and Lovich 2009). Finally, large numbers of turtles and turtle eggs can be easily collected from the field and maintained in captivity, which allows them to be excellent subjects for both field and laboratory research. Their natural history characteristics, life history traits, and experimental tractability make turtles ideal sentinel species for assessing and monitoring the effects of the Kingston, TN coal ash spill.

Although impacts of coal ash-derived trace elements are likely to manifest as direct reproductive effects, adult turtles may also experience other effects that could influence individual health. For example, exposure to ash-derived trace element contamination can cause liver necrosis in reptiles (Ganser et al. 2003). In addition, bioaccumulation of ash-derived trace elements can increase standard metabolic rate in amphibians (Rowe et al. 1998) and reptiles (Hopkins et al. 1999). In the absence of increased energy assimilation affected animals would have less energy available for activity, growth, and/or reproduction (Dunham et al. 1989, Congdon et al. 2001). Thus, the coal ash spill could cause indirect bioenergetic effects due to nutritional deficiencies, in addition to direct toxicological effects of exposure to ash-derived trace elements (Hopkins et al. 2004b). In addition, because females may excrete trace elements during egg-laying (e.g., Guirlet et al. 2008; but see Hopkins et al., 2013a) while males do not, the relative vulnerability of the sexes to trace element contamination may differ.

The goals of this study were to determine the spatial extent of trace element exposure in turtles living in areas of the Emory and Clinch Rivers impacted by the coal ash spill and determine whether exposure to ash-derived trace elements significantly affected capture rates, body sizes, and sex ratios of turtles among impacted and reference sites.

METHODS

SAMPLE COLLECTION

Our study focused on four turtle species at potentially high risk of trace element exposure: spiny softshell turtle (*Apalone spinifera*), snapping turtle (*Chelydra serpentina*), eastern musk turtle (*Sternotherus odoratus*), and pond slider (*Trachemys scripta*). *Apalone spinifera* and *C. serpentina* are large, long-lived carnivores that occupy high trophic levels, and should be at risk of bioaccumulation and biomagnification of trace elements (Meyers-Schone and Walton 1994, Bergeron et al. 2007, Hopkins et al. 2013b). *Sternotherus odoratus* is a small, primarily carnivorous generalist that has been shown to be at risk of trace element exposure because of its benthic feeding ecology (Bergeron et al. 2007, Hopkins et al. 2013b). *Trachemys scripta* is an intermediate-sized opportunistic generalist that is extremely abundant in the Tennessee River system and has been shown to accumulate and maternally transfer trace elements derived from coal ash (Nagle et al. 2001).

From April-July 2011 and April-August 2012, we trapped turtles in the vicinity of the Kingston, TN Fossil Plant using hoop traps baited with sardines and/or chicken. Traps were set in shallow-water areas (< 1 m deep) in microhabitats suitable for turtles. All hoop traps were fitted with Styrofoam to float traps during periods of high water, which allowed turtles continuous access to air regardless of surface water levels. Trapping was concentrated among sections of the Emory (river km 0.0-5.5) and Clinch (river km 0.0-7.0) Rivers (impacted by the coal fly ash spill) and within a section of the Tennessee River (river km 914-922), which was not impacted by the spill (Figure 1). Throughout the study, we generally maintained at least 15 traps at various sites along both the Emory and Clinch Rivers, and 15-20 traps at sites along the Tennessee River (45-50 total traps per day). Trap locations were documented using GPS and were recorded in field notebooks, along with the date and the times at which traps were set and checked. Traps were rebaited every three days, and were rotated among trapping locations depending upon trapping success.

Turtles were collected from traps daily, unless weather and flow conditions prevented river access. Trap contents were recorded and targeted species of turtles were placed in water-filled plastic tubs for transport back to a field laboratory. *T. scripta* was captured in large numbers (occasionally > 100/day); logistical considerations made it impractical to process all individuals of this species. In 2011, we processed all gravid female *T. scripta* but only one male or non-gravid female *T. scripta* from each river on each day of sampling. In 2012, we attempted to process the majority of captured *T. scripta*. Because sampling of *T. scripta* was relatively haphazard, we do not include this species in all statistical analyses (described below).

Turtles were processed at a laboratory facility in Kingston, TN. We measured turtle mass (g) with Pesola® scales (Baar, Switzerland), and carapace length (cm), carapace width (cm), and plastron length (cm) using forestry calipers. Turtles were individually marked for future identification using either PIT tags (*A. spinifera*) or three notches in the marginal scutes of the shell (all other species). We removed the top 2-3 mm of all claws (if present) on the right rear foot of every turtle for trace element analysis. We also sampled blood (0.5-1.0 ml) from *A. spinifera*, *C. serpentina*, *S. odoratus*, and *T. scripta*, using heparinized 1-ml tuberculin syringes fitted with 26.5-gauge hypodermic needles. We sampled blood from the cervical sinus of *S. odoratus* and *T. scripta*, and from the caudal vein of *A. spinifera* and *C. serpentina*. In addition to sampling blood and/or claws from all turtles, we took muscle biopsies from *C. serpentina* and shell samples from *A. spinifera.* Muscle biopsies (50-200 mg/wet weight) were taken from the ventral-lateral aspect of the tail of *C. serpentina* following administration of Lidocaine (Hopkins et al. 2013a). The biopsy site was then closed with two stitches of clear Polydioxanone monofilament (3/8 cm). We applied a topical anesthetic to reduce the risk of infection, and then coated the suture site with a liquid bandage (New Skin®). Shell

samples were taken from the right- posterior portion of the shell of *A. spinifera*. A triangular wedge (~1 cm wide, ~1 cm long, 0.05- 0.30 g) of tissue was cut from the shell with a scalpel. Bleeding was cauterized by application of silver nitrate. Removal of shell tissue not only allowed for trace element analysis, but also provided a secondary marker with which to identify recaptured turtles. Turtles were released at the point of capture the day after processing, or as soon as weather conditions permitted. Blood, claw, muscle, and shell samples were frozen at -20°C until submission to Dartmouth College for trace element analysis. Hereafter, we focus on trace-element concentrations in claws because this tissue is a relatively integrative measure of trace element exposure (Hopkins et al. 2013a, b). Identifying the relationships between trace-element concentrations among the different tissue types is a subject of ongoing investigation in our lab and will be completed once all trace element data have been received.

SAMPLE PROCESSING

Claw samples required no preparation at Virginia Tech and were shipped overnight on dry ice to the Trace Element Analysis Core at Dartmouth College. At Dartmouth College, claws were first washed to remove external contamination. Individual claws were transferred to a 7 ml polyethylene vial, 2 ml 1% solution of Triton X-100 was added and the vial was then placed in an ultrasonic bath for 20 minutes. The claw sample was washed five times with deionized water and then dried in the vial in a dry box.

Arsenic, Ba, Cd, Cr, Cu, Fe, Mn, Hg, Se, Sr, Tl, V, and Zn concentrations were quantified using Inductively Coupled Mass Spectrometry (ICP-MS) at the Trace Element Analysis Core at Dartmouth College. Dried tissues were stored at 4°C prior to sample preparation and analysis. Each sample was weighed into a pre-weighed VWR trace metal clean polypropylene centrifuge tube and 0.5 ml of 9:1 HNO_3:HCl (Optima Grade, Fisher Scientific) was added (0.25 ml for samples < 5 mg). Individual subsample weights were variable but were generally < 0.05 g. Tissue samples were prepared for acid digestion in batches of 100 samples along with five each of blank certified-reference material, and fortified blank quality control samples. All tubes were lightly capped and placed into a CEM MARS Express (Mathews, NC) microwave digestion unit for an open vessel digestion. A fiber optic temperature probe was placed into one of the sample tubes to provide temperature feedback to the MARS unit and the samples were heated to 95°C with a ramp to temperature of 15 minutes and held at temperature for 45 minutes. The samples were then allowed to cool and 0.1 ml of H_2O_2 (Optima Grade, Fisher Scientific) was added and the samples were taken through a further microwave heating program. The samples were then brought up to 10 ml (5 ml for < 5 mg samples) with deionized water (Element QPod, Millipore, Billarica, MA). All measurements were recorded gravimetrically.

We analyzed samples by collision cell ICP-MS (7700x, Agilent, Santa Clara, CA). Selenium (78) was measured in hydrogen mode (2.8 ml min^{-1}), Hg, Pb and Tl in no gas mode and all other analytes in He mode (4.8 ml min^{-1}). Analytical procedures followed the general protocols outlined in EPA 6020A; the instrument was calibrated with NIST-traceable standards and calibration was verified with a second source traceable standard. Reporting limits were checked after each calibration by running standards at 1, 2 and 3X the reporting limit. A continuing calibration check and blank was run every 10 samples. Analysis duplicates and spikes were run at a frequency of 1 duplicate and spike for each 20 samples.

Detection limits for each tissue sample varied because the mass of each tissue sample used in the analysis varied. If our analyses indicated that the concentration of an element in a given sample was below its detection limit, we assigned that element a concentration of half of its detection limit. Average detection limits for claw samples (mg/kg dry mass) for each element from *T. scripta* and *S. odoratus* are presented within Table 1. All trace-element concentrations are reported on a dry mass basis.

STATISTICAL ANALYSIS

In all parametric statistical tests, univariate normality and homoscedasticity of variance were assessed using Shapiro-Wilk Tests and normal probability plots, respectively. If necessary, data were log- transformed to improve normality and homoscedasticity of variance. All statistical tests were performed using R (R Core Development Team 2012) and significance was judged at $\alpha = 0.05$.

Trap effort (trap nights) was determined by calculating the elapsed time from trap-setting to trap-checking for each trap. We quantified daily trap success for turtle species by dividing the total captures of each species in a given trap by the amount of time since the trap was last checked. We compared mean daily trap success for each species among rivers using Kruskal-Wallis and Mann-Whitney tests.

For each species, we compared turtle morphology separately for each sex using river (Emory, Clinch, and Tennessee Rivers) of capture as a categorical treatment effect. We only included adults within this analysis because it is difficult to reliably sex juvenile turtles. We excluded recaptures from our analysis. Turtles were categorized as adults based on sizes at maturity obtained from literature reviews (Ernst and Lovich 2009). *Trachemys scripta* males and females were considered adult if they were ≥ 9.0 and 16.0 cm carapace length (CL), respectively. *Sternotherus odoratus* males and females were considered adult if they were ≥ 6.0 and 7.0 cm CL, respectively. *Chelydra serpentina* males and females were considered adult if they were ≥ 18.0 and 17.0 CL, respectively. Finally, *A. spinifera* males and females were considered adult if they were ≥ 130 grams and 18.0 cm plastron length, respectively. Adult turtle body mass and carapace length were compared among rivers separately for each sex using analyses of variance and Tukey's HSD. We determined whether the sex ratios of adult *S. odoratus, A. spinifera*, and *C. serpentina* differed among rivers with a Fisher's Exact Test. We again excluded recaptured individuals from this analysis; we also excluded *T. scripta* from this analysis because not all individuals of this species were marked.

Within this chapter, we report summary statistics for trace-element concentrations in claws from turtles captured in 2011 (Tables 2-5). Comprehensive statistics to examine trace-element concentrations in relation to sex, river, species, and size will be conducted once trace-element data are available from turtles collected in 2012.

RESULTS

TRAP EFFORT

Over the course of the two field seasons, we trapped the Clinch River, Emory, and Tennessee Rivers for 2,666, 2,861, and 2,921 trap nights, respectively. We recorded 5,077 captures in 2011 and 4,319 captures in 2012. Mean daily trap success for each species is reported in Table 6. Mean daily trap success did not differ among rivers for *S. odoratus* (H = 5.153, df = 2, P = 0.076). However, trap success did differ among rivers for *A. spinifera* (H = 44.988, df = 2, P < 0.001), *C. serpentina* (H = 6.423, df = 2, P = 0.04) and *T. scripta* (H = 27.024, df = 2, P < 0.001). For *A. spinifera*, daily trap success in the Tennessee was higher than in the Clinch (P < 0.001) and Emory Rivers (P < 0.001); daily trap success was also higher in the Emory River than the Clinch River (P = 0.004). For *C. serpentina*, daily trap success in the Tennessee River was higher than in the Emory River (P = 0.011). For *T. scripta*, daily trap success in the Clinch River was higher than in the Emory River (P < 0.001). Sex ratios did not differ among rivers for

adult *A. spinifera* or *S. odoratus*. Sex ratios for adult *C. serpentina* differed between the Clinch and Tennessee Rivers (Tables 7 and 8) due to a relatively high number of females captured in the Clinch River.

TURTLE MORPHOMETRICS

Neither carapace length (F = 1.570, df = 2,72, P = 0.215) nor body mass (F = 2.722, df = 2,72, P = 0.073) varied among rivers for male *A. spinifera* (Table 2). Likewise, neither carapace length (F = 0.984, df = 2,75, P = 0.379) nor body mass (F = 1.220, df = 2,75, P = 0.301) varied among rivers for female *A. spinifera*.

In contrast to *A. spinifera*, male *T. scripta* carapace length (F = 3.449, df = 2,817, P = 0.032) and body mass (F = 3.904, df = 2, 817, P = 0.021) differed among rivers (Table 9). Males were larger in the Tennessee River than the Clinch River for both carapace length (17.29 cm ± 0.19 vs. 16.69 cm ± 0.18, P = 0.049) and body mass (742.05 g ± 24.33 vs. 676.53 g ± 24.68, P = 0.027). Female *T. scripta* carapace length also differed among rivers (F = 3.862, df = 2,487, P = 0.022). Females were larger in the Tennessee (21.56 cm ± 0.13) than the Emory River (21.06 cm ± 0.18, P = 0.046).

Carapace length (F = 0.382, df = 2,89, P = 0.684) and body mass (F = 0.083, df = 2,89 P = 0.920) were similar among rivers for male *C. serpentina* (Table 9). Likewise, body mass of female *C. serpentina* did not vary among rivers (F = 1.730, df = 2,41, P = 0.190). Although our model indicated a marginally significant overall difference among rivers for female *C. serpentina* carapace length (F = 3.283, df = 2,41, P = 0.048), post-hoc pairwise comparisons did not reveal significant differences between rivers.

Male *S. odoratus* carapace length (F = 9.676, df = 2,666, P < 0.001) and body mass (F = 11.029, df = 2,666, P < 0.001) varied among rivers (Table 9). Male carapace length in the Tennessee (10.01 cm ± 0.06) was longer than in the Clinch (9.73 cm ± 0.08, P = 0.006) and the Emory (9.60 cm ± 0.06, P < 0.001) Rivers. Similarly, male body mass of *S. odoratus* in the Tennessee (148.91 g ± 2.55) was larger than in the Clinch (138.01 g ± 2.83, P = 0.007) and the Emory Rivers (130.84 g ± 2.24, P < 0.001). In contrast, female *S. odoratus* did not vary among rivers in terms of carapace length (F = 2.045, df = 2,385, P = 0.131) or body mass (F = 2.541, df = 2,385, P = 0.080)

DISCUSSION

Overall, our capture rates were similar to those of other studies comparing the effectiveness of hoop traps to other means of turtle capture (Gamble 2006, Sterrett et al. 2010). In addition, our capture rates highlight the substantial biomass that turtles maintain in aquatic systems (Iverson 1982), which allows excellent statistical power for comparisons of trace element exposure and effects over temporal and spatial scales. Although we do not statistically compare trace-element concentrations among rivers and species within this report, this will be completed once both years of trace-element data are available.

The lack of among-river differences in capture rates of *S. odoratus* suggests their abundances among rivers are comparable; however, there were among-river differences in capture rates of the three other target species. For *C. serpentina* and *A. spinifera,* mean rank trap success was higher in the Tennessee than in one or both of the impacted rivers. We suggest that ecological factors unrelated to the spill explain the differences in capture rates. For example, the aquatic habitats available on impacted rivers were generally closer to the main channel, rockier, and deeper than those available on the Tennessee River. *Apalone spinifera* and *C. serpentina* tend to prefer shallow backwaters and tributaries rather than deep main channel habitats (Ernst and Lovich 2009), and it is possible that the

habitats and associated prey available were less suitable for foraging in impacted rivers than in the Tennessee River. Similarly, although we documented a significant difference between the sex ratios of *C. serpentina* in the Clinch and Tennessee Rivers, we do not believe this result is due to factors related to the spill because of the absence of consistent trends among species and the unremarkable trace element concentrations accumulated by turtles in the impacted rivers. Rather, it is possible that female *C. serpentina*, which may have different patterns of space use and activity than males (Brown and Brooks 1993) are associated with specific but unquantified features of the sites we trapped on the Clinch River.

In general, male *S. odoratus* and both sexes of *T. scripta* from the Tennessee River were larger than individuals from the impacted Clinch and Emory Rivers. Although this result is consistent with the hypothesis that the coal ash spill could reduce growth rates in these two species from the Clinch and Emory Rivers over the two years prior to our sampling, we supply lines of evidence that suggest alternative explanations. First, body size differences among rivers were small (< 10%), and mean body sizes at all sites were well within the ranges reported for normal populations of these species (Ernst and Lovich 2009). Second, the embayments we sampled on the Tennessee River were generally larger in area, had less human development (i.e., houses, docks), and had more heavily-vegetated shorelines than did similar habitats on the Emory and Clinch Rivers. *Sternotherus odoratus* (and to a lesser extent, *T. scripta*) are known to forage heavily in areas with abundant plant detritus (Ernst and Lovich 2009), so the habitats present in the Tennessee River may allow greater foraging success than the habitats in the Emory and Clinch Rivers, regardless of spill conditions. Third, as mentioned previously, it is unlikely that trace-elements associated with the relatively recent 2008 Kingston coal fly-ash spill would have already led to observable differences in adult body size due to the long lifespans and slow growth rates of adult freshwater turtles. Finally, although *T. scripta* and *S. odoratus* body size varied among rivers, body size of *C. serpentina* and *A. spinifera* did not. The lack of a consistent trend among the species further supports the contention that differences in body size in *T. scripta* and *S. odoratus* are most likely not attributable to the coal ash spill.

We have not yet statistically analyzed trace element concentrations because we await the remaining analytical data. However, in general, trace-element concentrations were low (Tables 2-5) and likely below toxicity thresholds, based on reviews of other taxa (Beyer and Meador 2011). Trace-element concentrations could be relatively low because turtles simply did not accumulate substantial trace elements following the 2008 coal fly ash spill or because the extensive remediation efforts that occurred after the spill were effective at reducing the risk to these species. To help distinguish between these two possibilities, it may be helpful to compare trace element concentrations in turtles before mitigation was complete (i.e., 2009 and 2010) to concentrations after remediation efforts largely concluded (i.e., 2011 and 2012).

Qualitatively, some interesting trends emerged among trace element concentrations and will be the subject of future statistical investigation. For example, As concentrations in all species were higher in the Emory River than either the Tennessee or Clinch Rivers. Similarly, Se concentrations in all species were higher in the Clinch and Emory Rivers than the Tennessee. Trace element concentrations also appear to vary by species. For example, Ba and Sr concentrations were considerably higher in *S. odoratus* than in our other study species. This difference suggests there are interspecific differences in relative exposure to trace elements and we will be working to statistically evaluate these patterns when 2012 data are available.

In conclusion, we recorded more than nine thousand turtle captures and generated an extensive database that will allow us to statistically evaluate how trace-element concentrations are influencing turtles at both the individual and population level. Our preliminary analyses demonstrate that some study species were generally smaller and/or captured less frequently in the Clinch and Emory Rivers than our reference river while sex ratios did not differ among rivers (with one exception). Further research is required to statistically determine A) whether trace elements are

present in higher levels in turtles captured in the two rivers thought to be most influenced by the coal fly-ash spill, B) whether maternal transfer of trace elements is occurring, and C) whether this process is causing negative reproductive effects.

REFERENCES

Bergeron, C. M., J. F. Husak, J. M. Unrine, C. S. Romanek, and W. A. Hopkins. 2007. Influence of feeding ecology on blood mercury concentrations in four species of turtles. Environmental Toxicology and Chemistry **26**:1733-1741.

Beyer, W. N., and J. P. Meador, editors. 2011. Environmental Contaminants in Biota: Interpreting Tissue Concentrations. CRC Press, Boca Raton, FL.

Brown, G. P., and R. J. Brooks. 1993. Sexual and seasonal differences in activity in a northern population of snapping turtles, *Chelydra serpentina*. Herpetologica **49**:311-318.

Brown, G. P., C. A. Bishop, and R. J. Brooks. 1994. Growth rate, reproductive output, and temperature selection of snapping turtles in habitats of different productivities. Journal of Herpetology **28**:405-410.

Cherry, D. S., R. K. Guthrie, F. F. Sherberger, and S. R. Larick. 1979. The influence of coal ash and thermal discharges upon the distribution and bioaccumulation of aquatic invertebrates. Hydrobiologia **62**:257-267.

Congdon, J. D., J. L. Greene, and J. W. Gibbons. 1986. Biomass of freshwater turtles: a geographic comparison. American Midland Naturalist **115**:165-173.

Congdon, J. D., A. E. Dunham, W. A. Hopkins, C. L. Rowe, and T. G. Hinton. 2001. Resource allocation-based life histories: a conceptual basis for studies of ecological toxicology. Environmental Toxicology and Chemistry **20**:1698-1703.

Congdon, J. D., J. L. Greene, and R. J. Brooks. 2008. Reproductive and nesting ecology of female snapping turtles. Pages 123-134 *in* A. C. Steyermark, M. S. Finkler, and R. J. Brooks, editors. Biology of the Snapping Turtle. Johns Hopkins University Press, Baltimore, MD.

Dunham, A. E., B. W. Grant, and K. L. Overall. 1989. Interfaces between biophysical and physiological ecology, and the population ecology of terrestrial vertebrate ectotherms. Physiological Zoology **62**:335-355.

Eeva, T., E. Lehikoinen, and T. Pohjalainen. 1997. Pollution-related variation in food supply and breeding success in two hole-nesting passerines. Ecology **78**:1120-1131.

Eeva, T., M. Ryoma, and J. Riihimaki. 2005. Pollution-related changes in diets of two insectivorous passerines. Oecologia (Berlin) **145**:629-639.

Ernst, C. H. and J. E. Lovich. 2009. Turtles of the United States and Canada. 2nd edition. Johns Hopkins University Press, Baltimore, MD.

Galbraith, D. A., C. A. Bishop, R. J. Brooks, W. L. Simser, and K. P. Lampman. 1988. Factors affecting the density of populations of common snapping turtles, *Chelydra serpentina serpentina*. Canadian Journal of Zoology **66**:1233-1240.

Gamble, T. 2006. The relative efficiency of basking and hoop traps for painted turtles (*Chrysemys picta*). Herpetological Review **37**:308-312.

Ganser, L. R., W. A. Hopkins, L. O'Neil, S. Hasse, J. H. Roe, and D. M. Sever. 2003. Liver histopathology of the southern watersnake *Nerodia fasciata fasciata*, following chronic exposure to trace element-contaminated prey from a coal ash disposal site. Journal of Herpetology **37**:219-226.

Guirlet, E., K. Das, and M. Girondot. 2008. Maternal transfer of trace elements in leatherback turtles (*Dermochelys coriacea*) of French Guiana. Aquatic Toxicology **88**:267-276.

Hopkins, B. C., J. D. Willson, and W. A. Hopkins. 2013a. Mercury exposure is associated with negative effects on turtle reproduction. Environmental Science and Technology **47**:2416-2422.

Hopkins, W. A., C. Bodinof, S. Budischak, and C. Perkins. 2013b. Nondestructive indices of mercury exposure in three species of turtles occupying different trophic niches downstream from a former chloralkali facility. Ecotoxicology **22**:22-32.

Hopkins, W. A. 2000. Reptile toxicology: opportunities and challenges on the last frontier of vertebrate ecotoxicology. Environmental Toxicology and Chemistry **19**:2391-2393.

Hopkins, W. A., J. H. Roe, J. W. Snodgrass, B. P. Jackson, D. E. Kling, C. L. Rowe, and J. D. Congdon. 2001. Nondestructive indices of trace element exposure in squamate reptiles. Environmental Pollution **115**:1-7.

Hopkins, W. A. and C. L. Rowe. 2010. Interdisciplinary and hierarchical approaches for studying the effects of metals and metalloids on amphibians. Pages 325-336 *in* G. Linder and D. Sparling, editors. Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola, FL.

Hopkins, W. A., C. L. Rowe, and J. D. Congdon. 1999. Elevated trace element concentrations and standard metabolic rate in banded water snakes (*Nerodia fasciata*) exposed to coal combustion wastes. Environmental Toxicology and Chemistry **18**:1258-1263.

Hopkins, W. A., J. W. Snodgrass, J. H. Roe, B. P. Staub, B. P. Jackson, and J. D. Congdon. 2002. Effects of food ration on survival and sublethal responses of lake chubsuckers (*Erimyzon sucetta*) exposed to coal combustion wastes. Aquatic Toxicology (Amsterdam) **57**:191-202.

Hopkins, W. A., B. P. Staub, J. W. Snodgrass, B. E. Taylor, A. E. DeBiase, J. H. Roe, B. P. Jackson, and J. D. Congdon. 2004. Responses of benthic fish exposed to contaminants in outdoor microcosms- examining the ecological relevance of previous laboratory toxicity tests. Aquatic Toxicology (Amsterdam) **68**:1-12.

Iverson, J. B. 1982. Biomass in turtle populations - a neglected subject. Oecologia (Berlin) **55**:69- 76.

Longnecker, M. P., M. J. Stampfer, J. S. Morris, V. Spate, C. Baskett, M. Mason, and W. C. Willett. 1993. A 1-y trial of the effect of high-selenium bread on selenium concentrations in blood and toenails. American Journal of Clinical Nutrition **57**.

Meyers-Schone, L. and B. T. Walton. 1994. Turtles as monitors of chemical contaminants in the environment. Reviews of Environmental Contamination and Toxicology **135**:93-153.

Morel, F. M. M., R. J. M. Hudson, and N. M. Price. 1991. Limitation of productivity by trace metals in the sea. Limnology and Oceanography **36**:1742-1755.

Nagle, R. D., C. L. Rowe, and J. D. Congdon. 2001. Accumulation and selective maternal transfer of contaminants in the turtle *Trachemys scripta* associated with coal ash deposition. Archives of Environmental Contamination and Toxicology **40**:531-536.

R Core Team. 2012. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org.

Rowe, C. L., W. A. Hopkins, and J. D. Congdon. 2002. Ecotoxicological implications of aquatic disposal of coal combustion residues in the United States: A review. Environmental Monitoring and Assessment **80**:207-276.

Rowe, C. L., O. M. Kinney, R. D. Nagle, and J. D. Congdon. 1998. Elevated maintenance costs in an Anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. Physiological Zoology **71**:27-35.

Shukla, U. C., J. Singh, P. C. Joshi, and P. Kakkar. 2003. Effect of bioaccumulation of cadmium on biomass productivity, essential trace elements, chlorophyll biosynthesis, and macromolecules of wheat seedlings. Biological Trace Element Research **92**:257-273.

Sterrett, S. C., L. L. Smith, S. H. Schweitzer, and J. C. Maerz. 2010. An assessment of two methods for sampling river turtle assemblages. Herpetological Conservation and Biology **5**:490-497.

TVA. 2009. Corrective Action Plan for the TVA Kingston Fossil Plant Ash Release. Kingston, TN.

TABLES AND FIGURES

TABLE 1.

Mean detection limits (mg/kg; and standard errors) for claw samples of each of four turtle species included within this study.

	Trachemys	Sternotherus	Apalone	Chelydra
Arsenic	0.038 ± 0.002	0.109 ± 0.016	0.052 ± 0.007	0.020 ± 0.002
Barium	0.047 ± 0.003	0.147 ± 0.021	0.065 ± 0.009	0.025 ± 0.002
Cadmium	0.009 ± 0.001	0.027 ± 0.099	0.013 ± 0.002	0.005 ± <0.001
Chromium	0.563 ± 0.031	1.628 ± 0.238	0.784 ± 0.106	0.300 ± 0.025
Copper	0.281 ± 0.016	0.814 ± 0.119	0.392 ± 0.053	0.150 ± 0.013
Iron	9.377 ± 0.517	27.134 ± 3.975	13.067 ± 1.769	4.99 ± 0.420
Manganese	0.094 ± 0.005	0.282 ± 0.041	0.131 ± 0.018	0.055 ± 0.006
Mercury	0.188 ± 0.010	0.543 ± 0.079	0.283 ± 0.039	0.193 ± 0.024
Selenium	0.203 ± 0.018	0.505 ± 0.105	0.285 ± 0.056	0.106 ± 0.014
Strontium	0.038 ± 0.002	0.113 ± 0.016	0.052 ± 0.007	0.020 ± 0.002
Thallium	0.009 ± 0.001	0.027 ± 0.004	0.013 ± 0.002	0.005 ± <0.001
Vanadium	0.094 ± 0.005	0.271 ± 0.040	0.131 ± 0.018	0.050 ± 0.004
Zinc	2.583 ± 0.285	5.955 ± 0.953	4.573 ± 0.623	2.90 ± 0.367

TABLE 2.

Trace-element concentrations in claws of spiny softshell turtles (*Apalone spinifera*) captured in the Clinch, Emory, and Tennessee Rivers (2011). All sizes refer to carapace length (cm). All concentrations are mg/kg dry mass. Asterisks denote elements for which ≥ 50% of samples were below detection limits.

	Emory	Clinch	Tennessee
Total Males	10	7	12
Size Range (m)	16.5 - 18.8	15.6 - 20.3	14.8 - 32.3
Total Females	24	8	19
Size Range (f)	16.0 - 41.3	21.9 - 37.8	18.6 - 38.9
Aluminum	39.80 ± 5.10	48.25 ± 5.86	30.52 ± 7.58
Antimony*	0.02 ± < 0.01	0.02 ± 0.01	0.01 ± < 0.01
Arsenic	2.01 ± 0.37	0.72 ± 0.10	0.19 ± 0.02
Barium	2.72 ± 0.97	1.17 ± 0.18	1.36 ± 0.28
Beryllium*	0.01 ± < 0.01	0.01 ± < 0.01	0.01 ± < 0.01
Boron*	1.53 ± 0.40	1.37 ± 0.12	1.02 ± 0.08
Cadmium*	0.01 ± < 0.01	0.01± < 0.01	0.01 ± < 0.01
Chromium*	0.63 ± 0.12	1.00 ± 0.33	0.46 ± 0.08
Cobalt	0.07 ± 0.01	0.08 ± 0.01	0.06 ± 0.01
Copper	2.09 ± 0.33	1.14 ± 0.13	1.20 ± 1.6
Iron	198.46 ± 39.49	127.74 ± 19.04	158.41 ± 26.28
Lead	0.19 ± 0.02	0.17 ± 0.02	0.15 ± 0.02
Manganese	4.26 ± 0.69	6.29 ± 1.03	5.52 ± 0.89
Mercury	2.32 ± 0.39	1.34 ± 0.24	1.50 ± 0.19
Molybdenum*	0.05 ± 0.02	0.05 ± 0.02	0.02 ± < 0.01
Nickel*	0.31 ± 0.07	0.21 ± 0.02	0.20 ± 0.03
Selenium	2.85 ± 0.26	3.03 ± 0.34	1.03 ± 0.04
Silver	1.01 ± 0.57	5.68 ± 4.06	3.79 ± 1.90
Strontium	2.41 ± 0.85	1.54 ± 0.64	2.08 ± 1.09
Thallium*	0.01 ± < 0.01	0.01 ± < 0.01	0.01 ± < 0.01
Vanadium*	0.16 ± 0.03	0.13 ± 0.03	0.07 ± 0.01
Zinc	438.39 ± 12.19	430.40 ± 16.49	440.48 ± 12.18

TABLE 3.

Trace-element concentrations in claws of snapping turtles (*Chelydra serpentina*) captured in the Clinch, Emory, and Tennessee Rivers (2011). All sizes refer to carapace length (cm). All concentrations are mg/kg dry mass. Asterisks denote elements for which ≥ 50% of samples were below detection limits.

	Emory	Clinch	Tennessee
Total Males	16	13	20
Size Range (m)	18.2 - 38.7	20.8 - 36.4	16.5 - 38.8
Total Females	8	7	7
Size Range (f)	24.5 - 33.5	22.5 - 31.3	22.9 - 31.0
Total Unknown	2	2	1
Size Range (u)	13.3 - 14.2	8.7 - 12.1	17.3
Aluminum	63.36 ± 6.54	54.22 ± 5.33	78.39 ± 8.98
Antimony	0.01 ± < 0.01	0.01 ± < 0.01	0.01 ± < 0.01
Arsenic	1.64 ± 1.04	1.03 ± 0.19	0.49 ± 0.07
Barium	1.31 ± 0.42	1.15 ± 0.24	1.48 ± 0.24
Beryllium*	0.01 ± < 0.01	< 0.01 ± < 0.01	0.01 ± < 0.01
Boron*	0.50 ± 0.07	0.62 ± 0.09	0.54 ± 0.06
Cadmium	0.01 ± < 0.01	0.02 ± 0.01	0.01 ± < 0.01
Chromium	0.93 ± 0.16	0.74 ± 0.11	1.57 ± 0.20
Cobalt	0.11 ± 0.01	0.18 ± 0.05	0.13 ± 0.01
Copper	0.52 ± 0.08	0.65 ± 0.10	0.46 ± 0.04
Iron	303.39 ± 100.75	200.06 ± 31.20	366.94 ± 56.07
Lead	0.20 ± 0.01	0.23 ± 0.03	0.29 ± 0.03
Manganese	10.83 ± 2.20	6.36 ± 0.79	14.84 ± 2.99
Mercury	3.25 ± 0.34	3.98 ± 0.46	2.41 ± 0.35
Molybdenum	0.04 ± 0.02	0.08 ± 0.06	0.02 ± 0.01
Nickel	0.23 ± 0.06	0.27 ± 0.03	0.22 ± 0.03
Selenium	1.23 ± 0.06	1.77 ± 0.57	0.76 ± 0.03
Silver	0.06 ± 0.02	0.01 ± < 0.01	0.06 ± 0.03
Strontium	0.25 ± 0.05	0.56 ± 0.22	0.32 ± 0.05
Thallium*	< 0.01 ± < 0.01	< 0.01 ± < 0.01	< 0.01 ± < 0.01
Vanadium	0.17 ± 0.02	0.15 ± 0.01	0.19 ± 0.02
Zinc	350.04 ± 18.96	370.02 ± 15.97	360.10 ± 13.16

TABLE 4.

Trace-element concentrations in claws of eastern musk turtles (*Sternotherus odoratus*) captured in the Clinch, Emory, and Tennessee Rivers (2011). All sizes refer to carapace length (cm). All concentrations are mg/kg dry mass. Asterisks denote elements for which ≥ 50% of samples were below detection limits.

	Emory	Clinch	Tennessee
Total Males	12	8	13
Size Range (m)	9.0 - 10.6	8.7 - 11.3	8.1 - 11.7
Total Females	23	18	17
Size Range (f)	8.8 - 11.0	11.4 - 10.3	8.2 - 11.5
Aluminum	32.74 ± 5.27	24.45 ± 3.64	42.27 ± 7.30
Antimony*	0.02 ± < 0.01	0.03 ± < 0.01	0.04 ± 0.01
Arsenic	1.07 ± 0.19	0.77 ± 0.07	0.34 ± 0.05
Barium	45.14 ± 4.39	40.36 ± 6.55	33.62 ± 5.07
Beryllium*	0.01 ± < 0.01	0.01 ± < 0.01	0.02 ± 0.01
Boron*	1.74 ± 0.12	2.74 ± 0.63	3.87 ± 1.06
Cadmium*	0.02 ± 0.01	0.02 ± 0.01	0.01 ± < 0.01
Chromium	1.04 ± 0.34	0.92 ± 0.20	1.64 ± 0.34
Cobalt	0.09 ± 0.01	0.07 ± 0.01	0.16 ± 0.05
Copper	2.49 ± 0.34	2.33 ± 0.35	2.98 ± 0.75
Iron	115.36 ± 18.02	69.84 ± 8.48	203.22 ± 51.57
Lead	0.43 ± 0.05	0.38 ± 0.05	0.39 ± 0.05
Manganese	24.69 ± 7.59	13.96 ± 2.11	126.41 ± 71.32
Mercury	1.11 ± 0.09	0.96 ± 0.07	0.86 ± 0.11
Molybdenum*	0.04 ± < 0.01	0.06 ± 0.01	0.09 ± 0.02
Nickel*	0.36 ± 0.05	0.47 ± 0.10	0.75 ± 0.20
Selenium	1.21 ± 0.06	1.40 ± 0.09	0.74 ± 0.14
Silver	0.26 ± 0.19	0.06 ± 0.02	0.08 ± 0.04
Strontium	40.37 ± 4.68	44.88 ± 6.66	34.77 ± 5.38
Thallium	0.01 ± < 0.01	0.02 ± < 0.01	0.02 ± 0.01
Vanadium	0.21 ± 0.04	0.30 ± 0.04	0.24 ± 0.06
Zinc	491.15 ± 16.26	503.67 ± 16.86	541.46 ± 34.81

TABLE 5.

Trace-element concentrations in claws of pond sliders (*Trachemys scripta*) captured in the Clinch, Emory, and Tennessee Rivers (2011). All sizes refer to carapace length (cm). All concentrations are mg/kg dry mass. Asterisks denote elements for which ≥ 50% of samples were below detection limits.

	Emory	Clinch	Tennessee
Total Males	5	1	5
Size Range (m)	14.5 - 20.4	13.8	10.1 - 18.2
Total Females	23	28	30
Size Range (f)	14.7 - 26.7	20.0 - 23.5	19.6 - 24.5
Total Unknown	0	1	0
Size Range (u)	N/A	N/A	N/A
Aluminum	21.26 ± 3.43	17.95 ± 1.76	24.39 ± 5.73
Antimony*	0.01 ± < 0.01	0.01 ± < 0.01	0.01 ± < 0.01
Arsenic	4.46 ± 1.01	1.72 ± 0.22	0.39 ± 0.04
Barium	0.79 ± 0.21	0.78 ± 0.21	3.04 ± 1.02
Beryllium*	< 0.01 ± < 0.01	< 0.01 ± < 0.01	< 0.01 ± < 0.01
Boron*	0.94 ± 0.06	0.93 ± 0.06	0.95 ± 0.12
Cadmium*	0.01 ± < 0.01	0.01 ± < 0.01	0.01 ± < 0.01
Chromium*	0.47 ± 0.12	0.53 ± 0.12	0.69 ± 0.34
Cobalt	0.03 ± < 0.01	0.03 ± < 0.01	0.04 ± 0.01
Copper	1.40 ± 0.25	1.79 ± 0.26	2.13 ± 0.26
Iron	52.79 ± 6.96	52.46 ± 9.04	72.41 ± 11.44
Lead	0.10 ± 0.01	0.08 ± 0.01	0.13 ± 0.02
Manganese	1.81 ± 0.29	3.75 ± 1.53	5.43 ± 1.97
Mercury	1.65 ± 0.22	1.80 ± 0.17	1.40 ± 0.16
Molybdenum*	0.02 ± < 0.01	0.02 ± < 0.01	0.02 ± < 0.01
Nickel*	0.14 ± 0.01	0.17 ± 0.02	0.17 ± 0.02
Selenium	1.14 ± 0.13	0.98 ± 0.05	0.50 ± 0.02
Silver	0.03 ± 0.01	0.21 ± 0.12	0.07 ± 0.04
Strontium	1.09 ± 0.41	0.44 ± 0.22	5.16 ± 1.92
Thallium*	< 0.01 ± < 0.01	< 0.01 ± < 0.01	< 0.01 ± < 0.01
Vanadium	0.07 ± 0.01	0.06 ± < 0.01	0.07 ± 0.01
Zinc	325.82 ± 10.60	329.94 ± 10.37	329.56 ± 6.99

TABLE 6.

Catch per unit effort (average number of turtles per trap per day) for four freshwater turtle species captured from the Clinch, Emory, and Tennessee Rivers in 2011 and 2012.

	Emory	Clinch	Tennessee
Apalone spinifera	0.03 ± < 0.01	0.02 ± < 0.01	0.06 ± 0.01
Chelydra serpentina	0.02 ± < 0.01	0.03 ± < 0.01	0.03 ± < 0.01
Sternotherus odoratus	0.23 ± 0.01	0.25 ± 0.01	0.26 ± 0.01
Trachemys scripta	0.65 ± 0.03	0.87 ± 0.04	0.85 ± 0.04

TABLE 7.

Total individual adult males and females of three freshwater turtle species captured in 2011 and 2012 in two rivers (Clinch and Emory) impacted by a 2008 coal-fly ash spill in Kingston, Tennessee and a river (Tennessee) serving as a reference site. *Trachemys scripta* were not included in this analysis because we did not individually mark all captured turtles.

	Emory	Clinch	Tennessee
Chelydra serpentina			
Males	21	21	50
Females	9	18	17
Sternotherus odoratus			
Males	226	202	241
Females	123	135	130
Apalone spinifera			
Males	22	16	37
Females	32	12	34

TABLE 8.

Results (i.e., P values) from Fisher's exact tests used to determine whether sex ratios of three species of freshwater turtle (presented in Table 7) significantly differed among the Clinch, Emory, and Tennessee Rivers.

Apalone spinifera	Clinch	Tennessee
Emory	0.17	0.28
Clinch		0.82
Chelydra serpentina	Clinch	Tennessee
Emory	0.22	0.63
Clinch		0.04
Sternotherus odoratus	Clinch	Tennessee
Emory	0.21	0.94
Clinch		0.19

TABLE 9.

Mean morphology (and standard errors) of male and female freshwater turtles captured from the Clinch, Emory, and Tennessee Rivers in 2011 and 2012. Carapace length is expressed in centimeters and mass in grams.

	Emory	Clinch	Tennessee
Apalone spinifera			
Male			
n	22	16	37
Carapace Length	17.0 ± 0.38	17.66 ± 0.46	17.79 ± 0.25
Mass	443.18 ± 25.70	502.19 ± 35.27	526.16 ± 22.27
Female			
n	32	12	34
Carapace Length	35.02 ± 0.77	33.19 ± 1.48	33.93 ± 0.65
Mass	3922.97 ± 273.42	3385.42 ± 522.36	3438.82 ± 175.41
Chelydra serpentina			
Male			
n	21	21	50
Carapace Length	30.14 ± 1.29	29.35 ± 1.27	30.55 ± 0.74
Mass	6960.71 ± 708.28	6689.29 ± 786.89	6950.40 ± 453.01
Female			
n	9	18	17
Carapace length	28.23 ± 0.88	25.98 ± 0.86	28.20 ± 0.48
Mass	5327.78 ± 381.53	4491.11 ± 440.17	5335.29 ± 267.74
Sternotherus odoratus			
Male			
n	226	202	241
Carapace Length	9.60 ± 0.06	9.73 ± 0.08	10.01 ± 0.06
Mass	130.84 ± 2.24	138.01 ± 2.83	148.91 ± 2.55
Female			
n	123	135	130
Carapace Length	9.46 ± 0.07	9.41 ± 0.09	9.63 ± 0.08
Mass	140.47 ± 3.24	138.27 ± 3.62	147.5 ± 3.33
Trachemys scripta			
Male			
n	249	308	263
Carapace Length	17.18 ± 0.17	16.69 ± 0.18	17.29 ± 0.19
Mass	708.91 ± 17.92	676.53 ± 24.68	742.05 ± 24.33
Female			
n	132	140	218
Carapace Length	21.06 ± 0.18	21.09 ± 0.16	21.56 ± 0.13
Mass	1312.08 ± 32.01	1333.75 ± 30.87	1411.59 ± 25.32

FIGURE 1.

Segments of the Clinch, Emory, and Tennessee Rivers trapped for turtles in 2011 and 2012. Trapping occurred within river km 0.0-5.5, 0.0-7.0, and 914-922 of the Emory, Clinch, and Tennessee Rivers, respectively.